Serotypes, virulence genes and intimin types of Shiga toxin (verocytotoxin)-producing Escherichia coli isolates from minced beef in Lugo (Spain) from 1995 through 2003

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Abstract

Background: Shiga toxin-producing Escherichia coli (STEC) have emerged as pathogens that can cause food-borne infections and severe and potentially fatal illnesses in humans, such as haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS). In Spain, like in many other countries, STEC strains have been frequently isolated from ruminants, and represent a significant cause of sporadic cases of human infection. In view of the lack of data on STEC isolated from food in Spain, the objectives of this study were to determine the level of microbiological contamination and the prevalence of STEC O157:H7 and non-O157 in a large sampling of minced beef collected from 30 local stores in Lugo city between 1995 and 2003. Also to establish if those STEC isolated from food possessed the same virulence profiles as STEC strains causing human infections.

Results: STEC were detected in 95 (12%) of the 785 minced beef samples tested. STEC O157:H7 was isolated from eight (1.0%) samples and non-O157 STEC from 90 (11%) samples. Ninety-six STEC isolates were further characterized by PCR and serotyping. PCR showed that 28 (29%) isolates carried stx1 genes, 49 (51%) possessed stx2 genes, and 19 (20%) both stx1 and stx2. Enterohemolysin (ehxA) and intimin (eae) virulence genes were detected in 43 (45%) and in 25 (26%) of the isolates, respectively. Typing of the eae variants detected four types: γ1 (nine isolates), β1 (eight isolates), ε1 (three isolates), and θ (two isolates). The majority (68%) of STEC isolates belonged to serotypes previously detected in human STEC and 38% to serotypes associated with STEC isolated from patients with HUS. Ten new serotypes not previously described in raw beef products were also detected. The highly virulent seropathotypes O26:H11 stx1 eae-~/J1, O157:H7 stx1, stx2 eae-~/J1 and O157:H7 stx1 eae-~/J1, which are the most frequently observed among STEC causing human infections in Spain, were detected in 10 of the 96 STEC isolates. Furthermore, phage typing of STEC O157:H7 isolates showed that the majority (seven of eight isolates) belonged to the...
main phage types previously detected in STEC O157:H7 strains associated with severe human illnesses.

**Conclusion:** The results of this study do not differ greatly from those reported in other countries with regard to prevalence of O157 and non-O157 STEC in minced beef. As we suspected, serotypes different from O157:H7 also play an important role in food contamination in Spain, including the highly virulent serotype O26:H11 stx2, eae-β1. Thus, our data confirm minced beef in the city of Lugo as vehicles of highly pathogenic STEC. This requires that control measures to be introduced and implemented to increase the safety of minced beef.

**Background**

Shiga toxin-producing *Escherichia coli* (STEC), also called verocytotoxin-producing *E. coli* (VTEC), have emerged as pathogens that can cause food-borne infections and severe and potentially fatal illnesses in humans, such as haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS). STEC strains that cause human infections belong to a large number of O:H serotypes. Certain STEC strains belonging to serotypes O26:H11, O103:H2, O111:H8, O145:H28, and O157:H7 have been more frequently isolated from humans with severe illnesses. Furthermore, most outbreaks of HC and HUS have been attributed to strains of the enterohemorrhagic serotype O157:H7. However, as non-O157 STEC are more prevalent in animals and are food contaminants, humans are probably more exposed to these strains [1-3]. In Spain, STEC represent a significant cause of sporadic cases of human infection [3]. STEC isolates have caused eight outbreaks in Spain: six produced by the serotype O157:H7 (mainly of PT2), one by the serotype O26:H11, and one by the serotype O111:H-[-3].

Pathogenicity of STEC is associated with various virulence factors. The main factor is the capacity to form two potent phage-encoded cytotoxins called Shiga-toxins (Stx1 and Stx2) or verocytotoxins (VT1 and VT2) [1]. In addition to toxin production, another virulence-associated factor expressed by STEC is a protein called intimin encoded by the *eae* gene and responsible for intimate attachment of STEC to the intestinal epithelial cells, and which causes attaching and effacing (A/E) lesions in the intestinal mucosa [4,5]. The association between infections with intimin-positive STEC and severe illnesses in humans was demonstrated previously, but it was also shown that intimin is not essential for the virulence of certain STEC strains. At present, 21 (α1, α2, β1, ξR/β2B, δ/β2O, κ, γ1, γ2, θ, ε1, νR/ε2, ζ, η1, η2, t1, μR/μ2, λ, μB, νB, ξB and α) types and subtypes of intimins encoding in the *eae* gene have been identified [5-8]. It is discussed that different intimins may be responsible for different host- and tissue cell tropism [9]. A factor that may also affect virulence of STEC is the enterohemolysin (Ehly), also called enterohemorrhagic *E. coli* haemolysin (EHEC-HlyA), encoded by the *ehxλ* gene [10].

Ruminants, and especially cattle, represent one of the largest reservoirs of STEC, undercooked minced beef and raw milk being the major vehicles of STEC outbreaks [6,11-15]. The results of our previous studies indicated that STEC colonization is widespread among cattle in Spain. Between 1993 and 1995, 1,069 healthy cattle were examined for STEC colonization. STEC-positive animals were found in 95% of the farms examined and the estimated proportion of positive cattle in each farm ranged from 0 to 100%. The overall prevalence rates of non-O157 STEC colonization were estimated to be 37% in calves and 27% in cows. In a survey between 1998 and 1999, STEC O157:H7 was isolated from 55 (12%) of 471 feedlot calves (four to eight months of age). Although only a mean of three animals per herd were sampled, STEC O157:H7 isolates were detected in 32 (22%) of the 145 feedlots examined [11,13].

Our current investigation is the first large study in Spain on prevalence of STEC O157 and non-O157 in raw beef products. In view of the increasing importance of STEC as emerging food-borne pathogens, the high STEC prevalence in cattle and the lack of data from STEC isolated from food in Spain, the objective of this study were to determine the prevalence of STEC O157:H7 and non-O157 in a large sampling of minced beef collected from 30 local stores in Lugo city between 1995 and 2003. Another objective was to establish the serotypes, virulence genes and intimin types of those STEC isolated from minced beef to determine their potential pathogenicity to humans.

**Results and discussion**

In the present study STEC were detected in 95 (12%) of the 785 minced beef samples collected. STEC O157:H7 was isolated from eight (1.0%) samples and non-O157 STEC were isolated from 90 (11%) (Table 1). In three samples, both STEC O157:H7 and non-O157 (O8:H21, O22:H8, O110:H-) were isolated. The best results for detection and isolation of STEC were obtained when the cells were plated onto cefsime tellurite sorbitol MacConkey (CTSMAC) agar (18 h at 44°C) and MacConkey (MAC) agar (18 h at 44°C). The highest prevalence of STEC was detected in the samples with higher level of
fecal contamination. Thus, STEC were detected in 33% of minced beef samples with more than 999 E. coli per gramme (Table 2).

The comparison of data on STEC prevalence in food is subject to certain limitations. First, the use of different sampling methods on different types of products. Second, protocols for isolation and detection of these pathogens vary and are not uniform among and within laboratories. Third, most studies are focused on O157:H7, and thus underestimate STEC prevalence by ignoring the presence of non-O157 STEC. In any case, figures found in our laboratory do not differ greatly from those reported abroad (12% in New Zealand, 11% in Canada, 11% in the United Kingdom, 9% in Thailand) with studies carried out on the prevalence of both O157 and non-O157 STEC in minced beef [16-19]. Higher prevalences were reported in other countries (i.e. USA, Argentina, India). Thus, three main studies were carried out in Washington state with prevalences of 23% [20], 17% [21], and a quite different 1% [22]. In Argentina, Parma et al. [23] detected a prevalence of 30% in 50 minced beef samples. In India, Khan et al. [24] found 50% of 111 minced beef samples positive for stx by PCR.

In our study STEC O157:H7 was isolated from eight (1.0%) samples. A similar prevalence was detected in different countries: 1% and 0.4% in United Kingdom [25,26]; 1% in The Netherlands [27]; 0.4% and 2% in Italy [28,29]; 0.3% in Czech Republic [30]. In USA, prevalences reported varied from 0% [31], 0.7% [32] and 2.8% [33]. Higher prevalences were detected in Argentina 3.8% [34], China 5% [35], Perú 19% [36], and in Canada, with two studies of 2% and 29% of prevalence, respectively [32,37].

Analyzing Table 1, we can appreciate a longitudinal maintenance of the prevalence of non-O157 STEC in minced beef samples, with a decline prevalence occurred during 2002. However, it should be considered that only 20 samples were analysed during this year. The same happens with STEC O157:H7, whose prevalence fluctuates from 0% to 1.3%, with the exception of 1995, when it increased to 5%. The present study comprises data from 1995 to 2003, and data from this longitudinal period does not reflect any control measures to reduce contamination in raw minced beef sold in different stores from Lugo.

STEC strains that cause human infections belong to a large number of O:H serotypes (a total of 472 serotypes are listed in the authors’ website [38]. Most outbreaks of HC and HUS have been attributed to strains of the enterohemorrhagic serotype O157:H7 [39]. However, as Non-O157 STEC are more prevalent in animals and as contaminant in foods, humans are probably more exposed to these strains. Infections with some non-O157 STEC types, such as O26:H11 or H-(non-motile), O91:H21 or H-, O103:H2, O111:H-, O113:H21, O117:H7, O118:H16, O121:H19, O128:H2 or H-, O145:H28 or H- and O146:H21 are frequently associated with severe illnesses in humans, but the role of other non-O157 STEC types in human illnesses needs further examination [2,3].

In the present study we have characterized 96 STEC isolates (Table 3). These isolates belonged to 42 O serogroups, 61 O:H serotypes (including 10 new serotypes). The majority (68%) of STEC isolates belonged to serotypes previously found in human STEC and 38% to serotypes O5:H-, O8:H21, O22:H8, O26:H11, O26:H-, O76:H7, O91:H-, O103:H2, O103:H-, O111:H-, O112:H2, O113:H21, O118:H16, O145:H-, O157:H7, O174:H21, O174:H-, ONT:H4 and ONT:H3 (ONT = O not typeable) associated with STEC isolated from patients with HUS. By PCR the 96 STEC isolates showed that 28 (29%) carried stx1 genes, 49 (51%) stx2, and 19 (20%) both stx1 and stx2. Enterohemolysin (ehxA) and intimin (eae) virulence genes were detected in 43 (45%) and 25 (26%) of the isolates, respectively. A total of 71 different seropathotypes (associations between serotypes and virulence genes) were detected, being seropathotype O157:H7 stx1, stx2, eae-γ1, ehxA the most common (five isolates), followed by O8:H21 stx2 and ONT:H21 stx1 (four isolates, respectively), O26:H11 stx1, eae-β1, ehxA, and O64:H5 stx1 (three isolates, respectively). Like other authors [40], we

| Year | No. of samples analysed | STEC O157:H7 | Non-O157 STEC | Total STEC |
|------|------------------------|--------------|---------------|------------|
| 1995 | 58                     | 3            | 8             | 10         | 17%        |
| 1996 | 91                     | 0            | 8             | 8          | 9%         |
| 1997 | 173                    | 1            | 20            | 21         | 12%        |
| 1998 | 133                    | 1            | 18            | 18         | 14%        |
| 2001 | 80                     | 1            | 6             | 7          | 9%         |
| 2002 | 20                     | 0            | 1             | 1          | 5%         |
| 2003 | 230                    | 2            | 29            | 30         | 13%        |
| Total| 785                    | 8            | 90            | 95         | 12%        |
have observed that STEC isolated from beef and human STEC of the same serotype have similar known virulence-associated properties.

At least 90 phage types have been reported for STEC O157:H7 [41] but only seven of these (PT2, PT4, PT8, PT14, PT21/28, PT32 and PT54) accounted for the majority (>75%) of the human strains isolated in Europe and Canada [39]. Phage types PT2 and PT8 were predominant in human STEC O157:H7 strains in Spain as well as in many other European countries, including Belgium, Finland, Germany, Italy, England and Scotland; whereas PT14 was the most frequently isolated in Canada. Six different phage types were detected among the eight O157:H7 strains isolated in this study: PT2 and PT8 (two isolates, respectively); PT21/28, PT32, PT54, PT atypical (one isolate, each). Interestingly, seven of those eight isolates belonged to the most common phage types associated with severe human illnesses in Europe and Canada [39].

The eae gene, which has been shown to be necessary for attaching and effacing activity, encodes a 94- to 97-kDa outer membrane protein (OMP) which is termed intimin [4]. Numerous investigators have underlined the strong association between the carriage of eae gene and the capacity of STEC strains to cause severe human illnesses, especially HUS [1-3]. This important virulence gene was detected in 100% of STEC O157:H7 and in 19% of non-O157 isolates from beef in the present study. Nevertheless, production of intimin is not essential for pathogenesis, because a number of sporadic cases of HUS have been caused by eae-negative non-O157 STEC strains. Thus, STEC O104:H21 and O113:H21 strains lacking eae gene were responsible for an outbreak and a cluster of three HUS cases in USA and Australia, respectively [1,42].

Differentiation of intimin alleles represents an important tool for STEC typing in routine diagnostics as well as in pathogenesis, epidemiological, clonal and immunological studies [5,43-48]. 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 possible to differentiate at present 21 variants of the eae gene encoding 21 different intimin types and subtypes (α1, α2, β1, ξR/β2B, β/β2O, κ, γ1, γ2, θ, ε1, νR/ε2, ζ, η1, η2, t1, μR/τ1, λ, μB, νB, ξB and ο) [5,8,48]. In this study, four eae variants were differentiated: γ1 (in nine isolates), β1 (eight isolates), ε1 (three isolates), and θ (two isolates).

**Conclusion**

Seropathotypes O26:H11 stx1 eae-β1, O157:H7 stx1 stx2 eae-γ1 and O157:H7 stx2 eae-γ1 are the most frequently observed in STEC that cause human infections in Spain [3]. These highly virulent seropathotypes were detected in 10 of 96 STEC isolates characterized in the present study, confirming minced beef as potential source of STEC isolates pathogenic for humans and reinforces the need of HACCP (Hazard Analysis and Critical Control Point) programs all through food chain, from abattoirs to markets, in order to reduce contamination and consequently, illnesses related to minced beef. In addition, consumers should reduce their risk for STEC foodborne illnesses by following safe food-handling recommendations and by avoiding consumption of raw or undercooked meat products.

**Methods**

**Sample collection, culture, and STEC screening**

A total of 785 of samples of minced beef were collected from 30 local stores in Lugo city (Spain) between 1995 and 2003. A maximum of 10 samples for week were obtained at random from 10 markets located in different districts. Some stores from those 30 were sampled along the seven years, while others only during a period of the study. In most cases, stores had different meat suppliers.

All minced beef samples were packaged individually in sterile plastic bags, refrigerated for transportation to the laboratory, and processed within 2 h of collection. For isolation of STEC O157:H7 a 25 g portion of each minced beef sample was homogenized in a stomacher with 225 ml of buffered peptone water supplemented with vanco-
Table 3: Seropathotypes (serotypes and virulence genes) of STEC isolates (n = 96).

| Serotype       | Total | stx<sub>1</sub> | stx<sub>2</sub> | eae | ehxA | Serotype       | Total | stx<sub>1</sub> | stx<sub>2</sub> | eae | ehxA |
|----------------|-------|----------------|----------------|-----|------|----------------|-------|----------------|----------------|-----|------|
| O1:H20<sup>a</sup> | 1     | +              | -              | +   | (NR)<sup>d</sup> | +     | O112:H2       | 1     | +              | -              | +   |      |
| O1:H20<sup>b</sup> | 1     | +              | -              | +   |      | O113:H21      | 1     | -              | +              | -   | +    |
| O2:H8<sup>c</sup> | 1     | -              | +              | -   |      | O113:H21      | 1     | -              | +              | -   |      |
| O2:H27<sup>a</sup> | 1     | -              | +              | -   |      | O116:H16      | 1     | -              | +              | -   |      |
| O2:H32         | 1     | -              | +              | -   |      | O116:H21      | 1     | +              | -              | +   |      |
| O2:H<sup>a</sup> | 1     | -              | +              | -   |      | O118:H16      | 1     | +              | -              | (β1) | +    |
| O4:H4          | 1     |                |                | -   |      | O120:H10      | 1     | -              | +              | -   |      |
| O5:H<sup>b</sup> | 1     | +              | +              | (β1) | +    | O128:H31      | 1     | +              | -              | -   |      |
| O6:H10         | 1     | +              | -              | -   |      | O141:H19      | 1     | -              | +              | -   |      |
| O6:H49<sup>a</sup> | 1    | -              | +              | -   |      | O145:H<sup>b</sup> | 1    | +              | -              | (γ1) | +    |
| O8:H21         | 4     | -              | -              |      |      | O146:H21      | 1     | +              | -              | +   |      |
| O8:H<sup>a</sup> | 2     | +              | -              | +   |      | O146:H<sup>a</sup> | 1    | +              | -              | +   |      |
| O15:H16        | 1     | -              | +              | (β1) |      | O156:H8       | 1     | -              | +              | (NR) | -    |
| O15:H18<sup>c</sup> | 1  | -              | +              | -   |      | O157:H7      | 2     | -              | +              | (γ1) | +    |
| O17:H45        | 1     | -              | +              | -   |      | O157:H7      | 5     | +              | +              | (γ1) | +    |
| O20:H2<sup>c</sup> | 1  | +              | -              | -   |      | O162:H10      | 1     | +              | -              | -   |      |
| O21:H21        | 1     | +              | -              | -   |      | O166:H28      | 1     | +              | -              |     |      |
| O22:H8<sup>b</sup> | 2  | -              | +              | -   |      | O167:H2      | 1     | +              | -              | -   |      |
| O22:H8<sup>b</sup> | 2  | +              | +              | -   |      | O167:H2      | 1     | -              | +              | -   |      |
| O26:H11<sup>b</sup> | 3  | +              | -              | (β1) | +    | O168:H8      | 1     | -              | +              | -   |      |
| O26:H<sup>a</sup> | 1     | -              | +              | -   |      | O171:H2<sup>a</sup> | 2    | -              | +              | -   |      |
| O26:H42        | 1     | +              | -              | (β1) | +    | O174:H10      | 1     | +              | +              | (β1) | -    |
| O39:H21        | 1     | +              | -              | +   |      | O174:H21      | 1     | -              | +              | -   |      |
| O42:H21<sup>c</sup> | 1  | -              | +              | -   |      | O174:H<sup>b</sup> | 2    | -              | +              | -   |      |
| O54:H<sup>c</sup> | 1     | -              | +              | -   |      | OX178:H16      | 1     | -              | +              | -   |      |
| O64:H5<sup>c</sup> | 3     | +              | -              | -   |      | ONT:H4      | 1     | +              | -              | -   |      |
| O75:H8<sup>b</sup> | 1     | +              | -              | +   |      | ONT:H19      | 1     | -              | +              | -   |      |
| O75:H8<sup>b</sup> | 1     | -              | +              | -   |      | ONT:H21      | 1     | +              | -              | +   |      |
| O76:H7<sup>b</sup> | 1     | +              | -              | (NR) |      | ONT:H21      | 1     | +              | -              | +   |      |
| O77:H41<sup>a</sup> | 2  | -              | +              | -   |      | ONT:H21      | 4     | +              | -              | -   |      |
| O88:H8         | 1     | +              | -              | -   |      | ONT:H21      | 2     | -              | +              | -   |      |
| O91:H<sup>c</sup> | 1     | +              | -              | +   |      | ONT:H38      | 1     | -              | +              | -   |      |
| O103:H2<sup>b</sup> | 2  | +              | -              | + (c1) | +    | ONT:H<sup>c</sup> | 1    | -              | +              | -   |      |
| O103:H<sup>b</sup> | 1     | +              | -              | + (c1) | -    | ONT:H<sup>b</sup> | 1     | +              | -              | -   |      |
| O110:H<sup>c</sup> | 1     | +              | -              | +   |      | ONT:H<sup>b</sup> | 1     | -              | +              | -   |      |
| O111:H<sup>b</sup> | 2     | +              | -              | + (i) |      | ONT:H<sup>b</sup> | 1     | -              | +              | -   |      |

<sup>a</sup> Serotypes previously found in human STEC.
<sup>b</sup> Serotypes previously associated with human STEC strains that cause hemolytic uremic syndrome (HUS).
<sup>c</sup> New serotypes in STEC not reported in previous studies.
<sup>d</sup> Typing of eae (intimin) genes was carried out years after the isolation of the strains. Some of them had lost the viability, so eae typing could not be carried out (NR).
mycin 8 mg liter$^{-1}$, cefixime 0.05 mg liter$^{-1}$, and cefsulodin 10 mg liter$^{-1}$ (BPWvcc) and incubated for 6 h at 37°C. One millilitre of this enriched culture was added to 20 μl of magnetic beads coated with O157-antibodies (Dynabeads anti-E. coli O157, Dynal, Oslo) and the immunomagnetic separation (IMS) was performed according to the manufacturer’s instructions. The concentrated target cells were plated onto sorbitol MacConkey (SMAC) agar and on cefixime tellurite sorbitol MacConkey (CTSMAC) agar (18 h at 37°C). For isolation of non-O157 STEC a 25 g portion of each minced beef sample was homogenized in an stomacher with 225 ml of buffered peptone water and antibiotics (BPW) and incubated for 6 h at 37°C. The cells were plated onto CTSMAC and MacConkey (MAC) agar (18 h at 37°C and 44°C).

STEC were detected by PCR using specific primers for amplification of stx$\gamma_1$, stx$\gamma_2$, and eae genes (Table 4). For PCR, a loopful of bacterial growth taken from the first streaking area of the culture plates was suspended in 0.5 ml of sterile distilled water and boiled for 5 min to release the DNA. For each PCR-positive culture, 10 E. coli-like colonies obtained from MAC, SMAC and/or CTSMAC plates were analysed by PCR in order to obtain the STEC isolates for further characterization. If no positive single colony was found among the first 10 colonies, at least 40 more were tested. If still none of the assayed colony isolates was positive in the PCR reaction, the sample was reported as PCR positive without STEC isolation. Production of Shiga toxins (verocytotoxins) by PCR-positive isolates was confirmed by cytotoxicity tests on Vero and HeLa cells [13].

All STEC isolates were subsequently characterized biochemically with the API 20E system (bioMerieux, France) and serotyped. In samples from which all isolates were identical with respect to the profile of virulence genes and O:H serotypes, only one colony was selected. When one sample yielded colonies with different virulence genes or O:H serotypes, one of each was selected for further characterization.

**Enumeration of E. coli (MPN)**

Most probable number (MPN) determination was performed by standard method using Petrifilm™ Select E. coli Count Plates (3M Microbiology Products, USA), according to manufacturer’s instructions.

**Detection of virulence genes and typing of eae (intimin) genes by PCR**

The methodology used for the detection of virulence genes and typing of intimin genes into eae $\alpha_1$, $\alpha_2$, $\beta_1$, $\xi$/ $\beta$2B, $\delta$/ $\beta$2O, $\kappa$, $\gamma_1$, $\gamma_2$, $\theta$, $\epsilon$, $\nu$/ $\epsilon$/2, $\zeta$, $\eta_1$, $\eta_2$, $\iota$, $\mu$/ $\iota$/2, $\lambda$, $\mu$/8, $\psi$, $\beta$, and o has been described elsewhere [7,8,12,13,48]. Base sequences and predicted sizes of amplified products for the specific oligonucleotide primers used in this study are shown in Table 4. The oligonucleotide primers were designed by us according to the nucleotide sequences of the virulence genes. Isolates positive for 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 for distinguishing between eae-$\gamma_2$ and eae-$\delta$ genes, eae-$\delta$ and eae-$\kappa$, or eae-$\eta_1$ and eae-$\eta_2$. In those cases it was necessary to establish the nucleotide sequence of a fragment from the 3’ variable region of the eae gene. 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).

**Serotyping**

The determination of O and H antigens was carried out by the method described by Guine et al. [49] 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 the nonspecific agglutinins. The O antisera were produced in the LREC (Lugo, Spain) and the 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 (Statens Serum Institut).

**Phage typing of STEC O157:H7**

Phage typing was performed with the method of Khakria et al. [41] in the Centro Nacional de Microbiología (Madrid) [39,50] using phages provided by The National Laboratory for Enteric Pathogens, Laboratory for Disease Control, Ottawa, Ontario (Canada). The sixteen different phages used were capable of identifying 90 phage types.

**E. coli control strains**

E. coli strains used as controls were: EPEC-2348 (human, O127:H6, eae-$\alpha_1$), AEEC-H2498a (human, O125:H6, eae-$\alpha_2$), EPEC-337 (human, O111:H2, eae-$\beta_1$), EPEC-359 (human, O119:H6, eae-$\xi$/ $\beta$2B), EPEC-BL152.1 (human, O86:H34, eae-$\xi$/ $\beta$2O), EPEC-6044/95 (human, O118:H5, eae-$\kappa$), STEC-EDL933 (human, O157:H7, stx$\gamma_1$, stx$\gamma_2$, eae-$\gamma_1$), STEC-TW07926 (human, O111:H8, stx$\gamma_1$, stx$\gamma_2$, eae-$\delta$), STEC-VIB-286 (bovine, O103:H2, stx$\gamma_1$, eae-$\epsilon_1$), AEEC-6205a (human, O123:H19, eae-$\nu$/2), STEC-VTO-50 (ovine, O156:H7, stx$\gamma_1$, eae-$\epsilon_1$), AEEC-CF11201 (human, O125:H-, eae-$\eta_1$), H03/53199a (human, O107:H5, eae-$\kappa$), AEEC-7476/96 (human, O145:H4, eae-$\nu$/2), AEEC-217-2 (human, O101:H-, eae-$\mu$/2), AEEC-68-4 (human, O34:H-, eae-$\lambda$), EPEC-373 (human, O55:H51, eae-$\mu$B), AEEC-IH1229a (human, O10:H-, eae-$\lambda$), E. coli control strains were: EPEC-2348 (human, O127:H6, eae-$\alpha_1$), AEEC-H2498a (human, O125:H6, eae-$\alpha_2$), EPEC-337 (human, O111:H2, eae-$\beta_1$), EPEC-359 (human, O119:H6, eae-$\xi$/ $\beta$2B), EPEC-BL152.1 (human, O86:H34, eae-$\xi$/ $\beta$2O), EPEC-6044/95 (human, O118:H5, eae-$\kappa$), STEC-EDL933 (human, O157:H7, stx$\gamma_1$, stx$\gamma_2$, eae-$\gamma_1$), STEC-TW07926 (human, O111:H8, stx$\gamma_1$, stx$\gamma_2$, eae-$\delta$), STEC-VIB-286 (bovine, O103:H2, stx$\gamma_1$, eae-$\epsilon_1$), AEEC-6205a (human, O123:H19, eae-$\nu$/2), STEC-VTO-50 (ovine, O156:H7, stx$\gamma_1$, eae-$\epsilon_1$), AEEC-CF11201 (human, O125:H-, eae-$\eta_1$), H03/53199a (human, O107:H5, eae-$\kappa$), AEEC-7476/96 (human, O145:H4, eae-$\nu$/2), AEEC-217-2 (human, O101:H-, eae-$\mu$/2), AEEC-68-4 (human, O34:H-, eae-$\lambda$), EPEC-373 (human, O55:H51, eae-$\mu$B), AEEC-IH1229a (human, O10:H-, eae-$\lambda$).
| Gene  | Primer | Oligonucleotide sequence (5'-3') | Fragment size (bp) | Annealing temperature | Primer Coordinates<sup>a</sup> | Accession number |
|-------|--------|---------------------------------|-------------------|-----------------------|-------------------------------|-----------------|
| stx1  | VT1-A  | CGCTGAATGTCATTCGCTCTGC          | 302               | 55°C                  | 113–134                       | M17358          |
|       | VT1-B  | CGTGGTATAGCTAGCTGTCACC          |                   |                       | 394–414                       |                 |
| stx2  | VT2-A  | CTTCGAGATTTTAAATCCGAGCC         | 516               | 55°C                  | 50–69                         | M59422          |
|       | VT2-B  | CTTGCTTGACAGGTAGAAAGCCG         | 543–565           |                       |                               |                 |
| ehxA  | HlyA1  | GGTCGACGCAAAAAAGTGTAG           | 1.551             | 60°C                  | 238–259                       | X79839          |
|       | HlyA4  | TCTGCGCTGAGTTGTGTTGGTA          | 1767–1788         |                       | 631–654                       |                 |
| eae<sup>b</sup>  | EAE-1  | GGAAGCGGAAAGGTTAATCAGC         | 346               | 55°C                  | 957–976                       |                 |
|       | EAE-2  | GGCGTCATCATAGTCTTTC            |                   |                       | 1909–1928                     | AF022236        |
|       | EAE-3  | AAAACCGGGAAGATGACTTC            | 820               | 60°C                  | 2709–2728                     | AF500555        |
|       | EAE-4  | CACTTCTGCGATCTGAGCT             |                   |                       |                               |                 |
| eae-α1 | IH2498a | GACCTTTAGGTACATTAAGTAGC         | 517               | 60°C                  | 2099–2122                     | AF530555        |
|       | IH2498r | TCTGAGAAGAGGTTAATC             |                   |                       | 2597–2615                     |                 |
| eae-α2 | B1F    | CACAATTAATGACCGGGT             | 241               | 55°C                  | 2499–2512                     | AF530554        |
|       | B1R    | GCTTGATACACCGTGTGACT            | 2720–2739         |                       |                               |                 |
| eae-β1 | B2F    | TGAAGGGGGAACCCCTTGT             | 564               | 62°C                  | 2054–2073                     | AF530544        |
|       | B2R    | TTTCTTTTACGTGCAGAAAGGC         | 2596–2617         |                       |                               |                 |
| eae-κ/β2 | EAE-C1 | AGAAGCTTACGTGACTT              | 2303–2322         |                       | AF025311                     |
|       | EAE-C2 | AGAAGCTACTGTGACTTT              | 2697–2716         |                       |                               |                 |
| eae-ε1 | EAE-FB | AAAACCGGGAAGGTTAATC             | 722               | 66°C                  | 1909–1928                     | AF116899        |
|       | LP5    | AGCTCAGCAGGAGGATGAGGAAGGCGG    | 2605–2630         |                       |                               |                 |
| eae-νR/ε2 | EAE-E2F | AATACAGAAGTTAAGCATC         | 378               | 58°C                  | 2230–2248                     | AF530554        |
|       | EAE-E2R | AGCAACACTTATCCCTTGTC          | 2590–2607         |                       |                               |                 |
| eae-γ | Z1     | GGTAGGCGTTATCCTGCG             | 206               | 62°C                  | 2062–2079                     | AF449417        |
|       | Z2     | ATAGCAAGGGGTGGGTAAG            | 2250–2267         |                       |                               |                 |
| eae-η1/η2 | EAE-FB | AAAACCGGGAAGGTTAATC             | 702               | 60°C                  | 1899–1918                     | AJ308550        |
|       | ETA-B  | TAAGCAGCAGCATTCTGCTG           | 2582–2600         |                       |                               |                 |
| eae-η1 | ETA-FN | CGTTTTGGTTAATCGGCTAA           | 410               | 62°C                  | 1074–1095                     | AJ308550        |
|       | ETA-RN | GACTCAGCTGAAGTACG             | 1467–1483         |                       |                               |                 |
| eae-ι1 | IOTA-B | AAAAACCGGGAAGGTTAATC             | 651               | 55°C                  | 1909–1928                     | AJ308551        |
|       | IOTA-F | CGTCTGAGTTGACGTTG              | 2538–2559         |                       |                               |                 |
| eae-ι2 | IOTA2-F | CTTGTTAGAAAGGATGTTCAAC         | 1850–1870         |                       | AF530553                     |
|       | IOTA2-R | GGTCTTTGTGAAAGATGTTGC         | 2763–2785         |                       |                               |                 |
| eae-λ | 68.4F  | CGGTCAGCCTGGAAGGC              | 370               | 64°C                  | 2061–2079                     | AJ15409         |
|       | 68.4R  | AATACCGGAAGGAGGTTAATC         | 2410–2430         |                       |                               |                 |
| eae-μ | EAE-FB | AAAACCGGGAAGGTTAATC             | 655               | 60°C                  | 1909–1928                     | AJ705049        |
|       | FV373R | ACTCGATGAAAATCGTTTG            | 2541–2563         |                       |                               |                 |
| eae-νB | IH1229aF | CACAGTTAGAATGTTAACA     | 311               | 60°C                  | 2255–2275                     | AJ705050        |
|       | IH1229aR | CTACGATGAAAATCGTTTG           | 2545–2565         |                       |                               |                 |
| eae-ε2 | EAE-FB | AAAACCGGGAAGGTTAATC             | 468               | 66°C                  | 1909–1928                     | AJ705051        |
|       | B49R   | ACCACCGGGAAGGTTAATC             | 2355–2376         |                       |                               |                 |
| eae-ο | H2997f | AGCGTTAGCAGGCGGCGGTTGAT        | 271               | 60°C                  | 2203–2226                     | AJ876648        |
|       | IH2997f | CAACGGTAATTTTGTGTTCC           | 2454–2473         |                       |                               |                 |

<sup>a</sup> Location within eae gene. The oligonucleotide primers were designed by us according to the nucleotide sequences of the virulence genes.

<sup>b</sup>Universal oligonucleotide primer pair EAE1 and EAE-2 with homology to the 5' conserved region of eae gene (detects all types of eae variants described at the moment). Isolates positive for eae gene with EAE1 and EAE-2 primers were further analyzed with all different variant primers.
vB), STEC-B49 (bovine, O80:H1, stx₁, eae-ξB), AEEC-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% of agar.

Authors' contributions
AM, CL, and PJ carried out the isolation of the E. coli and performed the detection of virulence genes. MB, EAG and GD carried out the typing of the eae gene. JEB, MPA and MIB performed the serotyping. AE carried out the phage typing. JB carried out the design of the study and, together with AM, drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgements
This paper is dedicated to the memory of Dr. Enrique A. González, an eminent scientist, an excellent Professor of Microbiology and a very good friend. We thank to A. Román and M. Núñez for their collaboration in this study. We thank Monserrat Lamela for skillful technical assistance. This work was supported by grants from the Fondo de Investigación Sanitaria (grants FIS G03-025-COLURED-O157 and P100203), from the Xunta de Galicia (grants PGIDIT02BTF26101PR, PGIDIT04RAG261014PR, PGIDIT05STF26101PR, and PGIDIT06STL26101PR), from the Comisión Interministerial de Ciencia y Tecnología (grants CICYT-ALI98-0616 and CICYT-FEDER-1FD97-2181-C02-01), and from European Commission (FAIR programme grants CT98-4093 and CT98-3935). A. Mora, G. Dahbi and C. López acknowledge the Agencia Española de Cooperación Internacional (AECI) and the Xunta de Galicia for research fellowships.

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