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Shiga Toxin-producing *Escherichia coli* (STEC) O22:H8 isolated from cattle reduces *E. coli* O157:H7 adherence *in vitro* and *in vivo*

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Highlights

E. coli O22:H8 interfere with an effective colonization of cattle by *E. coli* O157:H7

E. coli O22:H8 has a stronger adherence to epithelial cell lines than *E. coli* O157:H7

E. coli O22:H8 has a enhanced biofilm production than that of *E. coli* O157:H7

In contrast to *E. coli* O157:H7, *E. coli* O22:H8 has the adhesin lpfAO113

Abstract

Problem addressed: Shiga toxin-producing Escherichia coli (STEC) are a group of bacteria responsible for food-associated diseases. Clinical features include a wide range of symptoms such as diarrhea, hemorrhagic colitis and the hemolytic uremic syndrome (HUS), a life-threatening condition.

Objective: Our group has observed that animals naturally colonized with STEC strains of unknown serotype were not efficiently colonized with *E. coli* O157:H7 after experimental infection. In order to assess the basis of the interference, three STEC strains were isolated from STEC persistently-colonized healthy cattle from a dairy farm in Buenos Aires, Argentina.

Methods and Results: The three isolated strains are *E. coli* O22:H8 and carry the *stx1* and *stx2d* genes. The activatable activity of Stx2d was demonstrated *in vitro*. The three strains carry the adhesins *iha*, *ehaA* and lpf_{O113}. *E. coli* O22:H8 formed stronger biofilms in abiotic surface than *E. coli* O157:H7 (*eae+, stx2+*) and displayed a more adherent phenotype *in*

vitro towards HeLa cells. Furthermore, when both serotypes were cultured together O22:H8 could reduce O157:H7 adherence *in vitro*. When calves were intragastrically pre-challenged with 10^8 CFU of a mixture of the three STEC strains and two days later challenged with the same dose of the strain *E. coli* O157:H7 438/99, the shedding of the pathogen was significantly reduced.

Conclusions: These results suggest that *E. coli* O22:H8, a serotype rarely associated with human illness, might compete with O157:H7 at the bovine recto-anal junction, making non-O157 carrying-calves less susceptible to O157:H7 colonization and shedding of the bacteria to the environment.

Keywords: *Escherichia coli*, STEC, EHEC; bovine, colonization, adhesion

1. Introduction

Shiga-toxin *Escherichia coli* is a major etiologic agent of diseases in humans, whose clinical spectrum includes diarrhoea, haemorrhagic colitis, and haemolytic uremic syndrome (HUS), the leading cause of chronic renal failure in children in Argentina and several other countries (Karch et al., 2005; Repetto, 2005). The most frequently associated serotype is O157:H7 (Rivas et al., 2006), although six non-O157 O groups (O26, O45, O103, O111, O121, and O145) have been identified by the Centers for Disease Control and Prevention (CDC) as being responsible for over 70% of non-O157 STEC-associated illness in the USA. (Brooks et al., 2005).

Ruminants are the primary reservoir of EHEC O157:H7 (Yoon and Hovde, 2008)and have been recently recognized as important reservoirs for other STEC serotypes, some of them associated with human disease (Parul et al., 2016). In Argentina, cattle have been identified as a source of non-O157 STEC and some of these strains have been previously

associated with hemolytic uremic syndrome (HUS), bloody and non-bloody diarrhea (Fernandez et al., 2013; Masana et al., 2011; Pizarro et al., 2013). Despite the carriage of Shiga toxins genes, posing a significant threat to human health, the combination with other virulence factors needs to be further assessed in order to evaluate the pathogenic potential of STEC strains. In this sense, the mechanism of STEC adherence to intestinal epithelium is highly relevant for the understanding of the STEC-bovine relationship and for the design of vaccines and other antibacterial strategies.

One of the main features of *E. coli* O157:H7 is the Locus of Enterocyte Effacement (LEE), which is a 35.6 kb pathogenicity island encoding a type three system (T3SS) apparatus, secreted proteins, an adhesin called Intimin and its translocated receptor Tir (Translocated Intimin Receptor) (Franzin and Sircili, 2015). These proteins are responsible for the formation of the attaching and effacing lesion and the effective colonization of cattle intestine (Dean-Nystrom et al., 2008). Despite the crucial role of Intimin in bacterial attachment, *E. coli* O157:H7 possesses many other proteins involved in adherence, some of them shared with non-O157 STEC. Among the fimbriae, the long polar fimbriae (Lpf) is relevant in this matter, which binds to extracellular matrix and is important for intestinal colonization *in vivo*. The hemorrhagic coli pilus (HCP) is a Type 4 pili (T4P) that has been described for several Gram-negative pathogenic bacteria and associated to several pathogenic processes. HCP is involved in adherence to bovine epithelium, biofilm formation and induction of proinflammatory cytokines and it has been tested as a vaccine component in goats (Zhang et al., 2014). Another virulence factor shared by O157 and non-O157 strains (Cadona et al., 2013) is the autotransporter EhaA. This protein has been

implicated in bacterial attachment to extracellular matrix proteins, such as laminin and collagen, (Wells et al., 2008) and abiotic surfaces, and in promoting adhesion to primary epithelial cells of the bovine terminal rectum(Easton et al., 2011). Besides adhesins shared between STEC O157 and non-O157, several other virulence factors have been described only in LEE (-) strains. For example, Sab, the 146-kDa STEC autotransporter contributing to biofilm formation confers adherence to human epithelial cells and mediates also biofilm formation (Herold et al., 2009). However, this autotransporter adhesin has a low prevalence among isolates (Buvens and Pierard, 2012; Monaghan et al., 2011). The main characteristics of the above-mentioned adhesins were reviewed by Farfan and Torres (2011). In addition, Iha, an outer membrane protein adhesin related to the iron-regulated gene A (IrgA) of *Vibrio cholerae*, has been reported in non-STEC uropathogenic *E. coli* (Tarr et al., 2000) as well as in over 70% of the LEE-negative STEC strains associated with human clinical cases examined in studies in Germany (Hauser et al., 2013) and Argentina (Galli et al., 2010). Iha is present in many enterobacteria and it is associated with both adhesion and iron uptake (Colello et al., 2016). Furthermore, the STEC autoagglutinating adhesin (Saa), is expressed by LEE-negative STEC (Caprioli et al., 2005) and homologues of saa were found in several unrelated LEE-negative STEC serotypes associated with human disease (Paton et al., 2001).

It is well demonstrated that the adherence site of *E. coli* O157:H7 in cattle is the recto-anal junction (RAJ), a tissue whose surface is formed by two cell types, columnar epithelial cell associated to lymphoid follicle and squamous epithelial (RSE) cells (Kudva et al., 2012b). It has been observed that the T3SS is required for O157 adherence to the follicle-associated

epithelium at the RAJ (Naylor et al., 2003) while they are not relevant in the adherence to the RSE cells (Kudva et al., 2012b). Similarly, non-O157 STEC are adherent to RSE even when anti-T3SS antisera is present (Kudva et al., 2012b).

Only few reports have demonstrated that concurrent shedding of more than one serogroup may occur in an individual animal, probably because the concurrence of various serotypes is not frequently searched (Pearce et al., 2004; Mercado et al., 2004; Cernicchiaro et al., 2013, Venegas-Vargas,et al., 2016).

We have previously observed that experimental colonization of cattle with *E. coli* O157:H7 was not effective when animals were naturally colonized with some unidentified non-O157 STEC strains. The aim of the present work was to isolate and characterize non-O157 STEC strains from non-O157 persistently colonized healthy cattle and to assess the ability of such strains to interfere with *E. coli* O157:H7 colonization both *in vitro* and *in vivo*.

2. Materials and Methods

2.1. Isolation of Shiga toxin-producing *E. coli*

Recto-anal mucosal swabs (RAMS) were taken from 28 healthy Holstein-Fresian male calves from a dairy farm in Buenos Aires, Argentina. Briefly, the swabs were cultured in 3 ml of Trypticase Soy Broth (TSB, Oxoid, Basingstoke, UK) for 18 h at 37°C and then streaked onto Sorbitol MacConkey agar (Oxoid, Basingstoke, UK). The confluent growth zone was analyzed by a multiplex PCR to detect the *stx1, stx2, eae y O157rbf* genes. This procedure was performed 4 times, every 2 weeks, and in those animals with at least two positive samplings for *stx1* and/or *stx2* but *eae* and *O157*-negative, the confluent zone

was re-isolated. Isolated sorbitol-fermenting colonies were identified as *E. coli* by biochemical tests (Edwards et al., 1956) and stored on glycerol 15% at -20°C for further assessment.

Escherichia coli O157:H7 438/99 belongs to INTA´s Culture Collection and it was isolated from cow faeces from a dairy farm in Buenos Aires province, Argentina. This strain has been previously used in experimental infection studies performed by our group (Vilte et al., 2011) and it was selected for spontaneous resistance to nalidixic acid. It possesses the genes for enterohemolysin, γ-intimin, EspA, EspB, Stx2, and the pO157 plasmid.

2.2. Detection of virulence factor genes by PCR

All *E. coli* isolates were screened for the presence of the genetic sequences of stx_1, stx_2, *eae* and rfb_{O157} by a multiplex PCR.Non-O157 *Stx*-positive strains were further analyzed to assess the presence of several other virulence factors including 1) toxins: subtilase cytotoxin (*subAB*), cytolethal distending toxins (*cdt*III-*cdt*IV), cytotoxic necrotizing factors (*cnf*I, *cnf*II), heat-stable enterotoxin (*STa*), hemolysin (*hlyA*); 2) Type three secretion system: intimin (*eae*); 3)Fimbriae: F41, F5, S fimbriae (*sfaD/E*), P fimbriae (*papC*), long polar fimbriae (lpfAO157/OI-141, lpfAO113, lpfAO157/OI-154); 4) adhesins: iron-regulated homologue adhesin (*iha*), afimbrial adhesin VIII (*afaE8*), CS31A adhesins (*clpG*); haemorrhagic coli pilus (*hcpA*); 5) siderophores: aerobactin (*iucD*); 6) biofilm formation: STEC autotransporter contributing to biofilm formation (Sab), STEC autoagglutinating adhesion (*Saa*), EHEC autotransporter (*ehaA*).

Subtyping of Stx variants was performed by PCR using the primers and conditions described by Scheutz (Scheutz et al., 2012). Primers for these virulence factors are shown in Table 1.

2.3. Serotyping

Serotyping was conducted with somatic and flagellar antisera of the Statens Serum Institute (Copenhagen, Denmark), according to (Orskov et al., 1984).

2.4. Isolation of mouse intestinal mucus

Intestinal mucus was isolated as described elsewhere (Wadolkowski et al., 1990a; Wadolkowski et al., 1990b). Briefly, 10 male and female BALB/c mice of 8 weeks of age and approximately 20 g of body weight were acquired from the Animal Facility of the Faculty of Veterinary Science of the University of Buenos Aires. Animals received food and water ad libitum and were housed under controlled conditions of light (12-h light, 12-h dark) and temperature (20–21ºC). Protocols were approved by the Committee for Care and Use of Laboratory Animals of Buenos Aires University (CICUAL).

One day before mucus extraction, mice were treated orally with streptomycin (5g/L). After the animals were euthanized intestinal fragments were removed, placed in ice-cold HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (2.6 g/liter) pH 7.4 and 5mm-segments were opened along the mesenteric border. Mucosa was then gently scraped into 10 ml of HEPES buffer, and mucus was clarified by centrifugation (once at 12,000 x g for 10 min and once at 27,000 x g for 15 min). Total protein content of the mucus was measured with a bicinchoninic acid protein assay kit (BCA, Thermo Scientific, USA).

2.5. Stx cytotoxicity on Vero cells

Escherichia coli strains were incubated overnight at 37°C and 200 rpm in 5 ml of LB broth and subsequently the culture supernatant was obtained by centrifugation and filtration (0.22 µm filters). Filtered culture supernatants were then incubated at 37ºC for 4 h with HEPES alone or in mouse intestinal mucus at a final concentration of 2 mg/ml and assayed as previously described (Pistone Creydt et al., 2004). Briefly, $4x10^4$ Vero cells grown in 96-well plates were treated for 72 h under growth-arrested conditions (serum-free medium) with filtered culture supernatant from different strains. At the end of the incubation time, plates were washed twice with PBS (145 mM NaCl, 10 mM NaH_2PO_4, pH 7.2) and incubated for 2 h with freshly diluted neutral red in PBS to a final concentration of 50 µg/ml. Cells were then washed with 1% $CaCl_2$ and 4% formaldehyde twice and then were solubilized in 1% acetic acid and 50% ethanol. Absorbance at 546 nm was read in an automated plate spectrophotometer. Results were expressed as percent viability, with 100% represented by cells incubated under identical conditions but without treatment. The 50% cytotoxic dose (CD_{50}) corresponded to the dilution required to kill 50% of Vero cells.

2.6. Antimicrobial susceptibility testing

Antimicrobial susceptibility patterns were established according to the Kirby-Bauer method for ampicillin, amikacin, colistin, chloramphenicol, gentamicin, nalidixic acid, ciprofloxacin, streptomycin, tetracycline, nitrofurantoin and trimethoprim-sulfamethoxazole, according to CLSI standards and methods (M100-Clinical Laboratory

Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing: Twenty-five Informational Supplement M100-S25. CLSI, Wayne, PA, USA, 2015).

2.7. Pulsed-field gel electrophoresis (PFGE)

Macrorestriction fragment analysis by PFGE was performed following the Standard Operating Procedure for PulseNet PFGE of *Escherichia coli* O157:H7, *Escherichia coli* non-O157 (STEC), *Salmonella* serotypes, *Shigella sonnei* and *Shigella flexneri* (CDC, www.cdc.gov/pulsenet/PDF/ecoli-shigella-salmonella-pfge-protocol-508c.pdf) using 6.76 sec – 35.38 sec as initial and final switching time for non-O157 STEC. A CHEF DR-III system (Bio-Rad Laboratories, Hercules, CA, USA) and *Xba*I (Promega Corp., Madison, WI, USA) restriction enzyme were used. *Salmonella* Braenderup H9812 (kindly provided by the Centers for Disease Control, Atlanta, GA, USA) was included as PulseNet size standard strain. Images of gels were obtained using a Gel Doc-IT 2000 (Bio-Rad Laboratories was used for gel images acquisition. Analysis of TIFF images was performed using the BioNumerics v 5.1 software package (Applied Maths, Kortrijk, Belgium).

All STEC O22 strains included in the National Database of non-O157 STEC were used for comparison purposes.

2.8. Growth curve

The strains were grown in Luria Bertoni broth (LB) (Sigma-Aldrich, Missouri, USA) overnight at 37 °C and then diluted 1:100 in LB and grown with continuous aeration at 150 rpm in triplicates. To measure bacterial growth, optical density at 600 nm was monitored every 30 min for the first hour and then every hour. Optical densities were analyzed with a longitudinal data model using Proc Mixed of SAS (SAS v.9.1; SAS Institute Inc., Cary NC,

USA). Fixed effects were strain, and linear, quadratic, and cubic terms for time of measure nested within strain. Error terms displayed heteroskedastic variance due to replicates and were fitted into the model by a diagonal covariance structure across replicates. Differences among strains at different times were tested as estimable linear contrasts (Searle, 1971). A correction of degrees of freedom for heterogeneous variance was implemented by the method of (Kenward and Roger, 1997) for all linear hypotheses. In all cases, P values of <0.05% were considered significant.

2.8. Biofilm formation assay

To assess biofilm formation, an overnight Luria Bertani broth culture of the strains grown at 37°C with shaking was diluted 1:40 on Dulbecco's modified Eagle's medium (DMEM) or TSB containing 0.45% glucose and 200 µL were inoculated into 96-well flat-bottom microtiter polystyrene plates (Becton Dickinson, Franklin Lakes, NJ). In addition, wells were filled with sterile media as the blank value. The plate was incubated overnight (18 h) at 37°C, washed twice with 200 µl PBS to remove unbound cells, and stained with 200 µl 0.1% crystal violet for 5 min. Unbound stain was removed by washing twice with PBS, and quantification of biofilm formation was carried out by the addition of 200 µl of 96% ethanol and measurement of the absorbance a*t* 540 nm (Herold et al., 2009; Wakimoto et al., 2004). The interpretation of biofilm production was done according to the criteria of (Stepanovic et al., 2007).

Data obtained were assessed for normality using the D'Agostino & Pearson omnibus normality test and a comparison between STEC strains with EHEC 438/99 and between

STEC strains was performed using one-way ANOVA. Significance was determined using Turkey's Multiple Comparison post test.

2.9. EHEC O157:H7 growth inhibition

In order to assess if the isolated STEC strains could inhibit EHEC O157:H7 growth the spot-on-lawn assay was performed. Briefly, an overnight culture of 438/99 was diluted to 0.5 McFarland Standard and spread onto a Mueller-Hinton agar plate (MHA, Oxoid, Basingstoke, UK) with a sterile cotton swab. STEC strains to be tested were also diluted to 0.5 McFarland Standard and 20 µl were spotted onto the surface of the agar plate. The plate was air-dried until the spots were dry and then incubated for 18 h at 37ºC. Plates were examined and a zone of inhibition around the test samples was considered as inhibition.

2.10. In vitro adherence

The ability of the isolated STEC strains to adhere to HeLa (ATCC-CCL2) and Caco-2 (ATCC-HTB-37) cell monolayers was assessed. Briefly, 12-well plates (Nest, USA) were inoculated with 2.5 10^5 cells per well and grown to 80% confluence at 37°C in 5% CO_2 in DMEM with 10% (vol/vol) heat-inactivated fetal bovine serum, 2 mM L-glutamine, penicillin (100,000 IU/liter) and streptomycin (100 mg/liter) (Amigo et al., 2015). Before use, the cells were washed three times with sterile phosphate-buffered saline (PBS; pH 7.4) and replenished with DMEM. The bacterial strains were grown in LB broth overnight at 37°C without aeration and 10^7 bacteria (MOI 40) were added to each well. The plates were incubated at 37°C with 5% CO_2 for 3 h and then washed twice with PBS in order to avoid Stx cytotoxicity. The cells were replenished with DMEM and incubated for additional 2h under

the conditions mentioned above. For *E*. *col* adherence quantification, the infected monolayers were washed three times with PBS, and the adherent bacteria were recovered with 1 mL of 0.1% Triton X100 in PBS and several dilutions were plated on LB agar plates with antibiotics. EHEC O157:H7 were plated in LB supplemented with nalidixic acid, while STEC O22:H8 were recovered in Streptomicyn-LB.

In competitive studies, both O22 strains and 438/99 were incubated together at a 1:1 ratio, recovered in SMAC plates and counted according to their sorbitol-fermenting abilities.

Data were expressed as the CFU (colony forming unit) from adhered bacteria from triplicate wells, and the mean of at least three separate experiments was used for the analysis. The statistical difference was expressed as the *P* value determined by a Kruskal-Wallis test, followed by the Dunn's multiple comparisons test.

2.11. In vivo assay

In order to evaluate if previous infection with the STEC O22:H8 strains could reduce *E. coli* O157:H7 colonization of calves after experimental challenge, 4 three-month-old male Holstein calves were obtained from a local dairy farm in Buenos Aires province. The animals were selected based on microbiological absence of Shiga-toxin producing *E. coli* by enrichment of rectoanal swabs, followed by PCR of the confluent zone to amplify *stx1* and *stx2* and specific antibodies against the carboxi-terminal fraction of Intimin gamma of *E. coli* O157:H7. Calves were housed in biosafety level 2 containment rooms at the Instituto Nacional de Tecnología Agropecuaria (INTA) Research Centre and fed alfalfa pellets, with free access to hay and water. All animal procedures were performed with the

approval of the Institutional Animal Care and Use of Experimentation Animals Committee (CICUAE) of the National Institution of Agricultural Research.

Animals were randomly divided into two groups of two animals each: Group 1 was intragastrically challenged with 10^8 CFU of a mixture of the three STEC O22:H8 strains isolated and two days later received 10^8 CFU of *E. coli* O157:H7 438/99 by the same route. Group 2 was only challenged with *E. coli* O157:H7 438/99 at the same time as the other animals, and it represents the positive control.

The magnitude of fecal excretion of viable *E. coli* O157:H7 was followed by culture of recto-anal mucosal swabs (RAMS), as previously described (Vilte et al., 2011; Vilte et al., 2012). RAMS were taken at-2, 0,2,4,6,9,12 and 15 days after 438/99 challenge.

Briefly, bacterial CFU/swab was determined by vortexing the swabs in TSB and plating serial dilutions on CT-SMAC. When direct cultures were negative, swabs were enriched at 37ºC for 18 h and 1 mL of this culture was subjected to *E. coli* O157 Immunomagnetic Separation (IMS) performed according to the manufacturer's instructions (Dynabeads anti-*E. coli* O157, Invitrogen Dynal AS, Oslo, Norway). The bead-bacterium mixture was plated on CT-SMAC supplemented with 20 ??g/mL nalidixic acid (Sigma, St. Louis, USA). Samples that resulted culture-positive by IMS were considered positive (10 CFU), while samples culture-negative by IMS were deemed negative. Non-sorbitol-fermenting colonies were tested for *E. coli* O157 LPS by latex agglutination (Oxoid, Basingstoke, UK) and confirmed by a multiplex PCR for stx_1, stx_2, *eae* and rfb_{O157} genes (Rabinovitz et al., 2012; Vilte et al., 2011; Vilte et al., 2012).

Fifteen days after challenge with *E coli* O157:H7, animals were euthanized and samples of cecum, ileum and recto-anal junction were cultured to detect *E. coli* O157:H7 after IMS as described above.

To perform the statistical analysis of *E. coli* O157:H7 shedding, data were transformed to achieve normality using the Inverse Hyperbolic Sine transformation. The model of analysis included fixed effects of treatments and time as a regression variable. Animals were treated as random and the repeated measures were fitted with an AR(1) heterogeneous covariance structure, using Proc Mixed of SAS.

3. Results

3.1. Isolation and characterization of Shiga toxin-producing *E. coli*

Three non-O157 strains were isolated from recto-anal swabs from healthy cattle persistently colonized with non-O157 STEC. They were named INTA 154, 155 and 182 after the animals they were isolated from. The three strains belonged to the O22:H8 serotype and were resistant to ampicillin, streptomycin and tetracycline. Stx subtyping revealed that the three *E. coli* O22:H8 isolates harbored the stx_{1c} and stx_{2d} subtype genes. Three of the several virulence factors tested were present in all the strains. These virulence factors were the iron-regulated homologue adhesion gene (*iha*), the EHEC autotransporter A (*ehaA*) and the long polar fimbriae (lpf_{O113}). The main phenotypic and genotypic characteristics of the strains are summarized in Table 2.

3.2. Subtyping by PFGE:

The isolated 154, 155, and 182 O22:H8 strains yielded two new *Xba*I-PFGE patterns named AREZJX01.0022, and AREZJX01.0023, respectively. These patterns were closely related, with at least 94.74% similarity, showing a difference in two bands among them (Fig. 1). The *Xba*I-PFGE patterns corresponding to O22:H8 STEC strains (7 from animal origin and one human), included in the National Database, were different from those detected in the present study, with less than 78% similarity.

3.2. Growth curve

The growth curves of the isolated O22:H8 strains and *E. coli* O157:H7 438/99 after 24 h are shown in Figure 2. The three O22:H8 strains had a statistically significant higher growth rate when compared to 438/99 at 1, 2, 3, 4 and 5 hours ($p < 0.05$), after the experiment started. However, no differences were found between the O22:H8 strains.

3.3. Biofilm formation

Biofilm formation was assessed on the three O22:H8 strains and compared to 438/99 on TSB as well as DMEM media supplemented with glucose. In both media, the three O22:H8 strains significantly formed more biofilm than the *E. coli* O157:H7 strain (Fig. 3). According to (Stepanovic et al., 2007), the three O22:H8 STEC strains were considered moderate biofilm producers on DMEM, whereas 154 and 155 were considered strong and 182 moderate on TSB. *E. coli* O157:H7 438/99 strain was considered weak and moderate on each media, respectively.

3.4. Stx cytotoxicity in STEC O22:H8 strains

The overnight culture supernatants from all STEC O22:H8 strains were toxic to Vero cells. The toxin level produced by each strain in absence of mucus (control) was lower than that

of STEC O157:H7 strain 438/99 (Table 3). Since the O22:H8 strains examined in this study possess the *stx1c genes* while 438/99 strain only has *stx*2a, these results can be attributed to the different toxin subtype. All STEC O22:H8 strains were further found to have active $Stx2d_{act}$, as the CD_{50} measured in the supernatants increased 15 to 95-fold after incubation with mouse intestinal mucus (Table3).

3.5. *E. coli* O157:H7 growth inhibition

No inhibition zone was observed in the plates were EHEC 438/99 was co-cultured with the STEC O22:H8 strains, suggesting that these strains do not inhibit *E. coli* O157:H7 growth (data not shown).

3.6. In vitro adherence

Adherence to epithelial HeLa (human cervix adenocarcinoma) and CaCo-2 (human colorectal adenocarcinoma) cell lines was assessed for the three O22:H8 strains and compared to *E. coli* O157:H7 438/99. Results are shown in Figure 4.

In vitro adherence of the STEC strains reveals a statistically significant increased adherence of the O22:H8 strains to HeLa cell, with strain 154 the most adherent. On CaCo-2 cells, 154 was the only strain with a more adherent phenotype compared to 438/99, whereas 155 and 182 had similar adherence properties, not statistically different when compared to the 438/99 strain.

When O22:H8 strains were cultured together with *E. coli* O157:H7 at a 1:1 ratio, significantly more CFU of O22:H8 were recovered from both HeLa and Caco-2 cell lines. Results are shown in Figure 5.

3.7. In vivo assay

In order to evaluate if the presence of STEC strains could interfere with *E. coli* O157:H7 colonization in experimentally challenged animals, a statistical analysis using a linear model for repeated measures (SAS) was performed which included fixed effects of treatments and time as a regression variable. Treatments were significantly different ($P < 0.0001$) whereas time was significant ($P= 0.0209$) and had a negative sign, which indicates a decreasing trend as time of observation went on. Treatment effects for the O22:H8 group were significantly different than the control, implying that *E. coli* O157:H7 shedding was significantly reduced after experimental challenge in 3-month-old calves previously challenged with the STEC O22:H8 strains, compared to the control group that only received *E. coli* O157:H7. Shedding dynamics are observed in Figure 6.

4. Discussion

During this study, three STEC strains were isolated from healthy cattle from a dairy farm in Buenos Aires, Argentina. The strains were identified as O22:H8, which is a serotype that had been previously reported in cattle in several countries (Blanco et al., 2004a; Ennis et al., 2012; Gonzalez et al., 2016) and rarely associated with human illness (Gould et al., 2013; Russmann et al., 1995).

In Argentina, it has been isolated from cattle (Blanco et al., 2004b), ground beef and hamburgers (Cadona et al., 2016), retail chicken products (Alonso et al., 2016) and even a domestic cat (Bentancor et al., 2007), suggesting that it is a circulating serotype in our country.

By *Xba*I-PFGE, the three strains showed more than 94% of similarity in their macrorestriction patterns. As they were isolated from different animals, it could be possible that the same or highly related strains were circulating among animals in the farm.

In the National Database (NDB), of non-O157 STEC, there are seven STEC O22:H8 strains isolated from bovine feces and carcasses in 2010 and one human diarrhea case in 2015. The low similarity between the patterns established in this study with those included in the NDB, suggests that different strains of this serotype are circulating in the country, distributed in animals more than in humans sources, as previously described by other authors (Alonso et al., 2016; Bentancor et al., 2012; Blanco et al., 2004b).

It has been reported that a part of the isolated STEC strains in cattle, including those identified here, do not possess the *eae* gene (Geue et al., 2002; Padola et al., 2004), and therefore other virulence factors are required adherence. The LEE negative O22:H8 serotype has been linked to super shedding (> log 4 CFU/g feces) in dairy cows over a twelve-month period, suggesting that adhesins other than Intimin play a role in the attachment *in vivo* and that these strain can persistently colonize cattle (Menrath et al., 2010) .

Despite several virulence profiles have been described for this serotype, in this study we have found that the O22:H8 strains isolated do not carry the Locus of Enterocyte Effacement (LEE) genes but harbor the *ehaA*, *ihA* and lpf_{O113} genes. These virulence factors are involved in adhesion and biofilm formation, like EhaA, which was identified as a novel autotransporter protein (AT) of enterohaemorrhagic *E.coli* (EHEC) O157:H7. The iron-

regulated homologue adhesin gene (*ihA*) codifies for a novel adherence-conferring molecule utilized by *eae*-negative STEC for adherence and colonization purposes instead of Intimin (Tarr et al., 2000) as well as the lpf_{O113} coding for the chromosomal fimbrial adhesin LpfAO113. The presence of LpfAO113 in O22: H8 strains might account for the increased adherence observed, since the O157:H7 strain, 438/99, does not have it. Despite Sab, another member of the AT family, has also been reported as a factor able to mediate adherence to human epithelial cells and biofilm formation, it was not detected in the strains isolated in this study. The three O22:H8 were also negative for SubAB, which is in agreement with previous reports (Bosilevac and Koohmaraie, 2011). Although many factors have been assessed throughout this work, several remain to be evaluated in order to explain the differences observed between strains.

The presence of the Stx2d subtype has been described in some O22:H8 strains (Beutin et al., 2007) and it has been shown that potentially highly pathogenic strains producing Stx2d activatable are *eae*-negative (Bielaszewska et al., 2006) although their relevance in human illnesses is moderate (Melton-Celsa et al., 2015). We have observed an increased cytotoxic activity of Stx2 after incubation with mucus, suggesting that this subtype might play an important role in pathogenesis.

Although the *in vitro* study was small, the statistic procedure applied was robust and suggests that infection of calves with the isolated strains of O22:H8 inhibits *E. coli* O157:H7 colonization. Since a epithelial cell line like HEp-2 seems to carry different adhesin receptors than RAJ cell types (Asper et al., 2007; Kudva et al., 2012a), a mixture of the

three STEC O22:H8 strains was used to challenge the animals to account for the differences observed *in vitro.*

These results suggest that *E. coli* O22:H8 are circulating among dairy cattle in our country. The strains isolated have several virulence markers, which represents a serious threat to human health. Furthermore, we have shown that these strains are able to interfere with *E. coli* O157:H7 adherence *in vitro* and *in vivo*. The molecular basis of the interference needs to be further assessed but so far, we have shown that O22:H8 strains are better fitted to compete with other strains given their growth rate, biofilm-forming abilities and adherence properties. As a next step the genome of O22:H8 isolates will be sequenced to identify probable adhesins.

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Figure Legends

Figure 1: Macrorestriction patterns of *Xba*I-PFGE of O22:H8 strains. Showing a dendrogram and including serotype and stx1 and stx2 genotype.

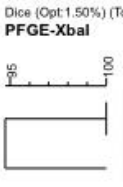
Figure 2: Growth curve of the STEC O22:H8 strains and the control EHEC O157:H7. * means statistical significance ($p < 0.05$).

Figure 3: Biofilm formation. *E. coli* O22:H8 (154, 155, 182) and O157:H7 (438/99) biofilm formation on DMEM and TSB. * shows significant differences ($p < 0.05$) with *E. coli* 438/99.

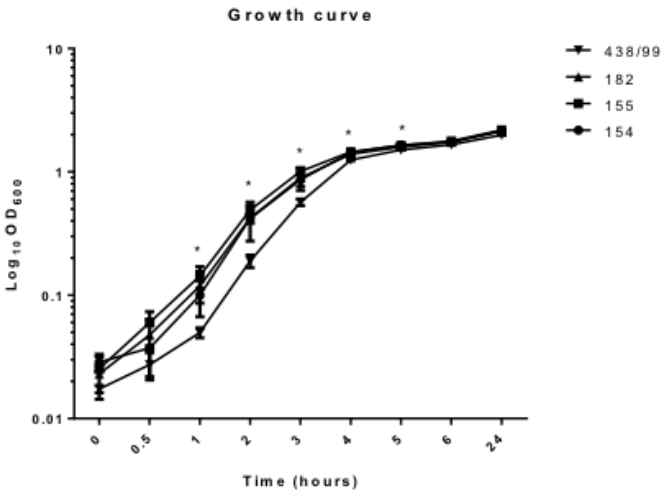
Figure 4: Adherence to cell lines. STEC 022:H8 (154, 155 and 182) and *E. coli* O157:H7 438/99 adherence to A) HeLa y B) Caco-2. * ($p < 0.05$) shows significant differences when compared to 438/99.

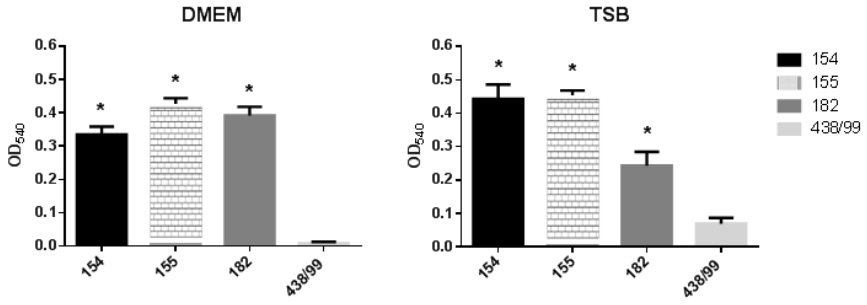
Figure 5: Adherence to cell lines competition. STEC 022:H8 and O157:H7 adherence to A) HeLa y B) Caco-2 when cultured together. *shows statistically significant differences ($p < 0.05$).

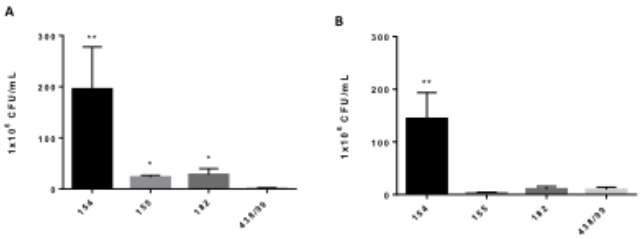
Figure 6: *E. coli* O157:H7 shedding up to 15 days of calves pre-challenged with a mixture of the STEC O22:H8 strains and the control group.

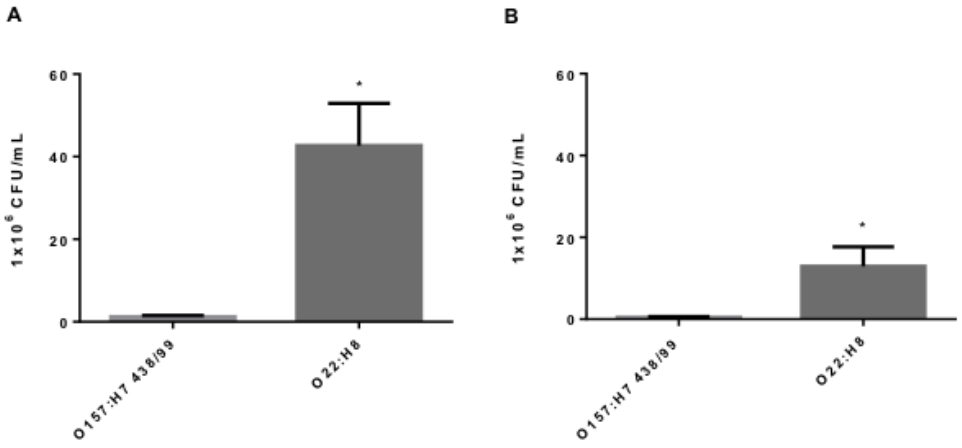


No.	Origin	*Xba*I-PFGE Pattern	Serotype	Genotype
154	Animal	AREZJX01.0022	*E. coli* O22:H8	stx_{1c} / stx_{2d}
155	Animal	AREZJX01.0022	*E. coli* O22:H8	stx_{1c} / stx_{2d}
182	Animal	AREZJX01.0023	*E. coli* O22:H8	stx_{1c} / stx_{2d}









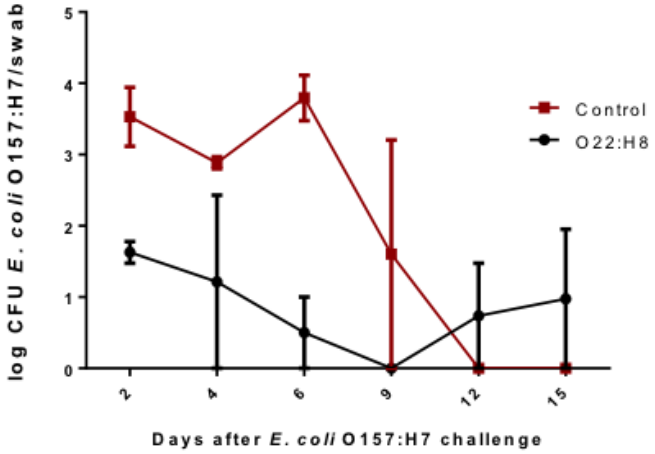


Table 1: Virulence factors, primer sequences and sizes of amplified DNA fragments used in this study.

Virulence factor	Primer	Sequence (5´3´)	Amplicon size (bp)	Reference
Intimin	EAE-1	GGAACGGCAGAGGTTAATCTGCAG	345	Blanco et al., (2004)
	EAE-2	GGCGCTCATCATAGTCTTTC		
Shiga toxin 1	Stx1-a	CAGTTAATGTGGTGGCGAAG	894	Olsvik and Strockbine (1993)
	Stx1-b	CTGCTAATAGTTCTGCGCATC		
Shiga toxin 2	Stx2-a	CTTCGGTATCCTATTCCCGG	478	
	Stx2-b	GGATGCATCTCTGGTCATTG		
rfbO157	O157F	CGGACATCCATGTGATATGG	258	Paton and Paton (1998)
	O157R	TTGCCTATGTACAGCTAATCC		
Cytotoxic necrotizing factor II	Cnf II F	TATCATACGGCAGGAGGAAGCACC	1243	Van Bost et al., (2003)
	Cnf II R	GTCACAATAGACAATAATTTTCCG		
Cytotoxic necrotizing factor I	Cnf_{1-2} F	TTATATAGTCGTCAAGATGGA	633	
	Cnf_{1-2} R	CACTAAGCTTTACAATATTGA		
Cytolethal distending toxin III	Cdt III F	GAAAATAAATGGAATATAAATGTCCG	558	
	Cdt III R	TTTGTGTCGGTGCAGCAGGGAAAA		
Cytolethal., distending toxin IV	Cdt IV F	CCTGATGGTTCAGGAGGCTGGTTC	350	
	Cdt IV R	TTGCTCCAGAATCTATACCT		
Aerobactin	IucD F	AAAACTGACATCGGATGGC	253	
	iucD R	GTATTTGTGGCAACGCAGAA		
S-fimbrial., adhesin	Sfa F	CTCCGGAGAACTGGGTGCATCTTAC	410	
	Sfa R	CGGAGGAGTAATTACAAACCTGGCA		

pili associated with pyelonephritis	pap F	GACGGCTGTACTGCAGGGTGTGGCG	328	
	pap R	ATATCCTTTCTGCAGGGATGCAATA		
Afimbrial., adhesin VIII	afa F	CTAACTTGCCATGCTGTGACAGTA	302	
	afa R	TTATCCCCTGCTTAGTTGTGAATC		
F17	F17 F	GCAGAAAATTCAATTTATCCTTGG	537	
	F17 R	CTGATAAGCGATGGTGTAATTAAC		
F5	F5 F	TATTATCTTAGGTGGTATGG	314	
	F5 R	GGTATCCTTTAGCAGCAGTATTTC		
F41	F41 F	GCATCAGCGGCAGTATCT	380	Franck et al., (1998)
	F41 R	GTCCCTAGCTCAGTATTATCACCT		
Heat-stable enterotoxin	Sta F	GCTAATGTTGGCAATTTTTATTTCTGTA	190	
	Sta R	AGGATTACAACAAAGTTCACAGCAGTAA		
Subtilisin	SubAB F	TATGGCTTCCCTCATTGCC	556	Paton and Paton (2005).
	SubAB R	TATAGCTGTTGCTTCTGACG		
Hemolysin	hlyA 1	GGTGCAGCAGAAAAAGTTGTAG	1551	Schmidt et al., (1995)
	hlyA4	TCTCGCCTGATAGTGTTTGGTA		
long polar fimbria O157/O154	O154-FCT	GCAGGTCACCTACAGGCGGC	525	Toma.et al., (2004)
	O154-RCT	CTGCGAGTCGGCGTTAGCTG		
long polar fimbria O157/O141	lpfO141-F	CTGCGCATTGCCGTAAC	412	Szalo et al., (2002).
	lpfO141-R	ATTTACAGGCGAGATCGTG		
long polar fimbria AO113	lpfA-F	ATGAAGCGTAATATTATAG	573	Doughty et al., (2002)
	lpfA-R	TTATTTCTTATATTCGAC		
Haemorragic coli pilus	hcpA-F	TCGCTAGTTGCTGACAGATTT	868	Hernandes et al., 2011
	hcpA-R	AATGTCTGTTGTGTGCGACTG		

STEC autotransporter [AT] contributing to biofilm formation	LH0147 F	GGTGGATACAGCAGGTAATG	163	Herold et al., (2009).
	LH0147 R	TATCTCACCACCTGCTATCG		
STEC autoagglutinating adhesin	SaaF	CCTCACATCTTCTGCAAATACC	688	Paton et al., (2001)
	SaaR	GTTGTCCTGCAGATTTTACCATCCAATGGACATG		
EHEC autotransporter	EhaA-u	AGGCATGAGACACGATC	500	Wu et al. (2010)
	EhaA-d	AAGTCGTGCCATTGAGC		
CS31A	clpG1	GGGCGCTCTCTCCTTCAAC	402	Bertin et al., (1998.
	clpG2	CGCCCTAATTGCTGGCGAC		
IrgA homologue adhesin	iha-I	CAGTTCAGTTTCGCATTCACC	1305	Schmidt et al., 2001
	iha-II	GTATGGCTCTGATGCGATG		

Table 2: genotypic characteristics of the strains

Strain	Biochemical identification	Sorbitol	Serotype		ATB[1]	Genotype[2]						
			O	H		0157	*Ea1*	*Hly*	*Stx*	*Lpf O113*	*Iha*	*ehaA*
438/99	*Escherichia coli*	-	157	7	R Nal	+	+	+	stx_{2a}/stx_{2c}	-	+	+
154	*Escherichia coli*	+	22	8	R Amp/Str	-	-	-	stx_{1c}/stx_{2d}	+	+	+
155	*Escherichia coli*	+	22	8	R Amp/Str	-	-	-	stx_{1c}/stx_{2d}	+	+	+
182	*Escherichia coli*	+	22	8	R Amp/Str	-	-	-	stx_{1c}/stx_{2d}	+	+	+

1. antibiotic resistance

2. genes and primers are defined in Table 1

Table3: Activation of Stx2d from STEC O22:H8 strains.

Strain	CD_{50} x 10^2 per mL		CD_{50} mucus/ CD_{50} Control
	Control	mucus	
O22:H8 155	0.11	10.5	95.5 ± 4.1*
O22:H8 154	0.11	10	91.0 ± 3.7*
O22:H8 182	1.5	22	15.2 ± 2.7†
O157:H7 438/99	2	2	1.0 ± 0.5

Ratios of cytotoxicity (CD50) of culture supernatant from STEC strains after incubation with intestinal mucus compared to the cytotoxicity without mucus (CD50 Control). The ratios are the averages from 2-3 experiments. Mean statistical significance (*$p < 0.01$ and †$P < 0.05$) with O157:H7 438/99.