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# Efficacy of a recombinant Intimin, EspB and Shiga toxin 2B vaccine in calves experimentally challenged with *Escherichia coli* O157:H7

Luisina Martorelli [a], Nicolás Garimano [b], Gabriela A. Fiorentino [c], Daniel A. Vilte [a], Sergio G. Garbaccio [a], Stefanie A. Barth [d], Christian Menge [d], Cristina Ibarra [b], Marina S. Palermo [c], Angel Cataldi [e,*]

[a] *Instituto de Patobiología, Centro de Investigación en Ciencias Veterinarias y Agronómicas, Instituto Nacional de Tecnología Agropecuaria (INTA), Hurlingham, Argentina*
[b] *Laboratorio de Fisiopatogenia, Departamento de Fisiología, Instituto de Fisiología y Biofísica Bernardo Houssay (IFIBIO Houssay-CONICET), Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina*
[c] *Laboratorio de Patogénesis e Inmunología de Procesos Infecciosos, Instituto de Medicina Experimental, (IMEX), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)-Academia Nacional de Medicina, Buenos Aires, Argentina*
[d] *Friedrich-Loeffler-Institut/Federal Research Institute for Animal Health, Institute of Molecular Pathogenesis, Jena, Germany*
[e] *Instituto de Biotecnología, Centro de Investigación en Ciencias Veterinarias y Agronómicas, Instituto Nacional de Tecnología Agropecuaria, Hurlingham, Argentina*

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ABSTRACT

*Escherichia coli* O157:H7 is a zoonotic pathogen of global importance and the serotype of Shiga toxin-producing *E. coli* (STEC) most frequently associated with Hemolytic Uremic Syndrome (HUS) in humans. The main STEC reservoir is cattle. Vaccination of calves with the carboxy-terminal fraction of Intimin γ (IntC280) and EspB can reduce *E. coli* O157:H7 fecal shedding after experimental challenge. Shiga toxin (Stx) exerts local immunosuppressive effects in the bovine intestine and Stx2B fused to *Brucella* lumazine synthase (BLS-Stx2B) induces Stx2-neutralizing antibodies. To determine if an immune response against Stx could improve a vaccine's effect on fecal shedding, groups of calves were immunized with EspB + IntC280, with EspB + IntC280 + BLS-Stx2B, or kept as controls. At 24 days post vaccination calves were challenged with *E. coli* O157:H7. Shedding of *E. coli* O157:H7 was assessed in recto-anal mucosal swabs by direct plating and enrichment followed by immunomagnetic separation and multiplex PCR. Calves were euthanized 15 days after the challenge and intestinal segments were obtained to assess mucosal antibodies. Vaccination induced a significant increase of IntC280 and EspB specific antibodies in serum and intestinal mucosa in both vaccinated groups. Antibodies against Stx2B were detected in serum and intestinal mucosa of animals vaccinated with 3 antigens. Sera and intestinal homogenates were able to neutralize Stx2 verocytotoxicity compared to the control and the 2-antigens vaccinated group. Both vaccines reduced *E. coli* O157:H7 shedding compared to the control group. The addition of Stx2B to the vaccine formulation did not result in a superior level of protection compared to the one conferred by IntC280 and EspB alone. It remains to be determined if the inclusion of Stx2B in the vaccine alters *E. coli* O157:H7 shedding patterns in the long term and after recurrent low dose exposure as occurring in cattle herds.


## 1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC), a subset of Shiga toxin-producing *E. coli* (STEC), is a globally important zoonotic pathogen capable of causing Hemorrhagic Colitis (HC) and Hemolytic Uremic Syndrome (HUS) in humans. HUS is often described as an epidemic disease of low incidence in industrialized countries such as USA, Canada and Japan (1–3 cases per 100,000 children under 5 years) [1]. However, Argentina is the country with the highest incidence per year in the world, with 12 to 15 cases per 100,000 children under 5 years old. HUS is the leading cause of acute renal failure in Argentinian children and the second cause of chronic renal failure, and is also responsible for 20% of kidney transplants in children and adolescents [2,3]. Human infection usually results from the consumption of fecal-contaminated foods containing EHEC. The main sources of infection are insufficiently cooked meat, unpasteurized milk, dairy products manufactured with unpasteurized milk, horticultural products contaminated by irrigation or fertilizers, and contaminated water. It can also be transmitted from person to person [1].

The main reservoir of STEC are ruminants, mainly bovine. EHEC O157:H7 preferentially colonizethe lymphoid follicle-dense

* Corresponding author at: Instituto de Biotecnología, INTA, Los Reseros y Nicolás Repetto, CP 1686 Hurlingham, Argentina.
*E-mail address:* Cataldi.angeladrian@inta.gob.ar (A. Cataldi).

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mucosa at the terminal rectum and the recto-anal junction (RAJ) [4]. The bacterium is shed to the environment intermittently through faecal matter [5], and occurs for longer periods and with a greater number of bacteria shed in young and weaning calves than in adult animals. EHEC O157:H7 has been found in dairy cattle and beef cattle, both grazing and feedlot [5–7].

*E. coli* O157:H7 is characterized by the possession of a number of virulence traits that confer pathogenicity and colonization properties. Among the first, Shiga toxins (Stx), which can be type 1 or 2, are the principal virulence factors implicated in human disease [8–10]. Despite the fact that cattle do not display any clinical symptoms that relate to EHEC carriage, bovine intestinal epithelial cells as well as peripheral and intraepithelial lymphocytes express functional Stx receptors [11–13]. Stx1 is considered an immunomodulating agent in cattle, blocking the activation and proliferation of bovine PBMC and mucosal lymphocytes *in vitro* [14,15]. Furthermore, an altered cytokine expression pattern has been observed in these cellular populations upon incubation with the toxin [14,16]. Infections with STEC can suppress the development of specific cellular immune responses during the early phase of immune activation in cattle, and this immunosuppression has been linked to Stx2 [17]. Stx2 has also been related to increased intestinal colonization by *E. coli* O157:H7 in mice [18].

The adhesion to intestinal mucosa and the "attaching and effacing" (A/E) lesion relies on a large pathogenicity island called "locus of enterocyte effacement" (LEE), which is a sequence of 35.6 Kb with 5 operons [19–21] that encodes for a type three secretion system (T3SS), i.e., a series of T3SS components, gene regulators and effectors proteins, and the protein responsible for intimate adherence (Intimin) to enterocytes along with its translocated receptor (Tir) [22].

Various *E. coli* O157:H7 virulence factors are capable of inducing an immune response in cattle during natural as well as experimental infections. It has been shown that calves respond serologically to LEE-encoded proteins such as Intimin, EspA and EspB, after an experimental infection with *E. coli* O157:H7 [23]. Antibodies against these proteins have also been found in people infected with EHEC and enteropathogenic *E. coli* (EPEC) [24,25] and in colostrum and milk from naturally infected cows [26].

Vaccination of cattle with bacterial colonization factors has been suggested as a strategy to reduce STEC colonization of the bovine gastrointestinal tract. Several experimental vaccines have been developed, many of them based on LEE secreted proteins, Tir and Intimin; O157 lipopolysaccharide, siderophores and porin receptors have also been tested with variable results [27–33]. We, along with other groups, have demonstrated that vaccination of calves with T3SS injection apparatus proteins results in reduced excretion of EHEC O157:H7 after experimental challenge [27,30,34,35]. The vaccine tested by our group included the recombinant carboxy-terminal fraction of Intimin γ and EspB with an oily adjuvant and calcitriol. Vaccinated calves showed high titers of serum IgG against both antigens after the first dose and specific IgA increased in saliva, but not in faecal matter. We verified a statistically significant reduction in faecal excretion of EHEC O157:H7 in the vaccinated group compared to the control. This protective effect was observed both at the level and frequency of excretion [27]. These results show that vaccination with T3SS recombinant proteins is a good strategy to reduce faecal shedding. However, vaccine formulations need to be further optimized in order to improve protection.

We have previously observed a specific and neutralizing response against Stx2 upon addition of BLS-Stx2B to the previously mentioned experimental vaccine [36]. The aim of the present study was to assess the ability of the 3-antigen formula to reduce EHEC carriage after experimental challenge. Also, several aspects of the immune response were evaluated.

## 2. Materials and methods

### 2.1. Animals

Fifteen 4-month-old conventionally reared Holstein Friesian male calves were obtained from a dairy farm in Buenos Aires Province, Argentina and housed at the Instituto Nacional de Tecnología Agropecuaria (INTA) Research Centre. Animals were selected on the basis of absence of Shiga toxin-producing *E. coli* carriage, assayed by enrichment of recto-anal mucosal swabs streaked onto sorbitol McConkey agar. The confluent growth zone was used as template to perform multiplex PCR to amplify *stx1*, *stx2*, *eae* and $O157_{rbf}$ [37–39]. Furthermore, the selected animals had low levels of serum antibodies against the carboxy-terminal fraction of Intimin γ and EspB (OD < 0.2 under the conditions explained below). Calves were fed alfalfa pellets, with free access to hay and water and treated prophylactically upon arrival with 1% Ivermectin to control intestinal nematodes. All animal experiments were performed with the approval of the Institutional Animal Care and Use of Experimentation Animals Committee (CICUAE) of the INTA in BSL2 containments facilities for large animals. One calf from the 2-antigen vaccinated group died 9 days after the challenge presumably due to pneumonia. It was excluded from all the analysis from that day onwards.

#### 2.1.1. Production of recombinant E. coli O157:H7 proteins

The coding sequences of EspB and IntC280 from the bovine *E. coli* O157:H7 strain 146N were cloned in pRSET-A vector (Invitrogen Corp., Carlsbad, CA) and expressed in *E. coli* BL21 (DE3)/pLysS, as described previously [26]. Briefly, the amino terminal-His-tagged proteins were purified from the lysates by affinity chromatography on Nickel-agarose columns (ProBond nickel-chelating resin; Invitrogen Corp.), eluted under denaturing conditions and dialyzed against PBS at pH7.4.

The B subunit of Stx2 was cloned upstream to the *Brucella* lumazine synthase (BLS) gene and the recombinant protein was expressed as described elsewhere [40]. This antigen was kindly provided by Fernando Goldbaum (Fundación Instituto Leloir, Buenos Aires, Argentina).

Recombinant Stx2B and a small fragment of A subunit were concomitantly expressed as previously described [41]. The A subunit fragment and the B subunit are expressed independently as separate polypeptides. The B subunit is fused to polyhistidine and was purified by Nickel agarose column. This antigen was used in anti-Stx2B ELISA, whole blood re-stimulation and re-stimulation of peripheral blood mononuclear cells (PBMC).

### 2.2. Immunization protocol

Calves were randomly separated into 3 groups and vaccinated according to the following scheme: non-vaccinated control (n = 5): PBS; Group 3Ag (n = 5): IntC280 + EspB + BLS-Stx2B; Group 2Ag (n = 5): IntC280 + EspB. The immunization scheme consisted on the application of 2 doses, 15 days apart (day 0 and day 15 post vaccination, dpv), with 100 μg of IntC280, 100 μg EspB and 300 μg of BLS-Stx2B by intramuscular route. The antigens were diluted in 1 mL of PBS and emulsified in 1 mL of mineral oil-based adjuvant (Montanide ISA206, Seppic, France). The control group was vaccinated only with PBS emulsified in the adjuvant.

### 2.3. Challenge

Ten days after the booster, animals were challenged intragastrically with $10^9$ CFU of *E. coli* O157:H7 strain 438/99 in 15 mL of sterile PBS. An overnight culture of the challenge strain was diluted

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