January 2004

Decreased shedding of *Escherichia coli* O157:H7 by cattle following vaccination with type III secreted proteins

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Potter, Andrew A.; Klashinsky, Sandra; Li, Yuling; Frey, Elizabeth; Townsend, Hugh; Rogan, Dragan; Erickson, Galen E.; Hinkley, Susanne; Klopfenstein, Terry J.; Moxley, Rodney A.; Smith, David R.; and Finlay, B. Brett, "Decreased shedding of *Escherichia coli* O157:H7 by cattle following vaccination with type III secreted proteins" (2004). *Papers in Veterinary and Biomedical Science*. 93. [https://digitalcommons.unl.edu/vetscipapers/93](https://digitalcommons.unl.edu/vetscipapers/93)
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1. Introduction

Enterohemorrhagic Escherichia coli (EHEC) is an important zoonotic pathogen of humans, causing severe diarrhea (hemorrhagic colitis) and in a small percentage of cases, haemolytic-uremic syndrome (HUS). There are approximately 75,000 infections and 61 deaths/year in the USA [1]. Unfortunately, antibiotics increase the risk of HUS [2], and there are no current therapies for EHEC human infections other than general supportive measures [3]. EHEC serotype O157:H7 causes 85–95% of the cases of HUS in North America [4], and is also the leading cause of HUS in Europe [3].

Cattle are an important reservoir for E. coli O157:H7 and many human infections are attributable to contact with contaminated meat or other sources, such as water, fruit or vegetables [4]. This organism can be found in 80% of some populations of live cattle [5], and 49% of beef carcasses [6], respectively. It does not appear to cause disease in adult cattle, although it can cause diarrhea in neonates [7]. Serologic evidence suggests that most calves are exposed to E. coli O157:H7 [8]. The number of animals shedding the organism in their feces is usually higher in the summer months [9], which correlates with an increased incidence of human disease. Shedding is intermittent, but it is not known if the organism remains in the intestines of cattle during periods of time when it cannot be recovered from feces.

Since cattle and their products are associated with the majority of cases of E. coli O157:H7 infection in humans, they represent an attractive target for pre-slaughter intervention as a means of reducing risk to humans. A number of approaches are being studied to reduce levels of the organism in cattle, including animal management practices such as chlorination of water [10], modifications to animal feed [11–13], the use of probiotics [14], and bacteriophage therapy [15]. Other vaccinations are also being investigated [16–18].

Proteins secreted by the type III system play a role in colonization of non-bovine hosts by E. coli O157:H7 [19],
and it is likely they are also required for colonization of the bovine intestine. One virulence determinant, Tir, is integrated into the host cell membrane where it serves as the receptor for the bacterial outer membrane protein, intimin [19]. Tir-intimin binding is essential for bacterial adherence to host cells [19–21]. Proteins secreted via the type III pathway, including Tir, EspA (which forms a linkage between bacterium and host cell), and EspB (which forms a pore in the host cell), are recognized by sera from convalescent individuals [22]. Dean-Nystrom et al. [20] demonstrated that intimin is required for the development of attaching-effacing (A/E) lesions in neonatal calves, and that pedestals (protrusions beneath adherent bacteria mediated by type III effectors such as Tir) are present in infected ileal tissue. Baehler and Moxley [23], by inoculating explants prepared from 18-month-old slaughtered steers, demonstrated that adult bovine colonic and rectal epithelia are susceptible to E. coli O157:H7 induced A/E lesions. Subsequent studies, based on experimental inoculation of adult cattle, demonstrated that expression of intimin [24] and Tir [25] are required for E. coli O157:H7 intestinal colonization. A study in swine demonstrated the potential for protection against A/E lesions and intestinal colonization by vaccination with intimin [26]. Collectively, these observations suggest that the type III secreted proteins are attractive targets for vaccine development.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The E. coli O157:H7 strain used for the production of type III secreted proteins and experimental infection was originally obtained from Li et al. [22] and Tarr et al. [27] and the tir mutant was described by Li et al. [22]. Recombinant E. coli K12 strains used for the production of Tir and EspA were as described [22].

2.2. Preparation of type III secreted proteins and vaccine formulation

Supernatant proteins [containing type III secreted proteins (Esps and Tir)] were prepared from E. coli O157:H7 precisely as described [22], and formulated with the adjuvant VSA3 [28] such that the protein concentration was either 25 or 100 μg/ml.

2.3. Vaccination and experimental infection of cattle

Calves and yearling cattle were obtained from farms in Saskatchewan, Canada and were housed at the University of Saskatchewan. They were fed a barley-based finishing ration with free choice roughage. All animals were screened prior to immunization for existing serum antibody titres against EHEC secreted proteins as well as shedding of E. coli O157:H7. Animals which had existing titres or which were shedding the organism at any point prior to experimental challenge were excluded from the study and were moved to separate housing to avoid cross-contamination. Cattle were immunized with 2 ml of each vaccine formulation delivered subcutaneously in the neck. In all experiments, a control vaccine group that received a formulation containing adjuvant only was included. Animals were challenged 2 weeks following the last immunization with 10⁸ CPU of E. coli O157:H7 by oral-gastric intubation and fecal shedding of the organism was monitored for 14 days. In the first experiment described below, eight seronegative 6-month-old calves were vaccinated twice with 2 ml of the vaccine formulation (100 μg/ml) by the subcutaneous route, while an equal number of age-matched calves received a placebo containing adjuvant but no antigen. Bacteria were detected by direct plating of fecal samples which had been resuspended in saline on Sorbitol MacConkey agar supplemented with cefixime and tellurite [29]. A second vaccine trial was designed in which three groups of yearling (adult) cattle were immunized as described above three times with 50 μg of supernatant (n=13), 50 μg of secreted proteins from a tir mutant [19] (ΔTir, n=10) or a placebo (n=25) on days 0, 21, and 35. Fecal samples from yearling cattle were cultured after immunomagnetic enrichment [29 and 30] as well as direct plating. The immune response following immunization was measured using serum samples taken at the time of each vaccination by an enzyme-linked immunosorbent assay [31] and by Western blotting [22]. Differences in outcome variables between or among vaccine groups were assessed using the Wilcoxon Rank Sum Test or the Kruskal–Wallis ANOVA, as appropriate. These differences were considered significant whenever P<0.05. This model typically results in measurable shedding of the organism for a period of approximately 60 days. All animal experiments were conducted in accordance with the guidelines established by the Canadian Council for Animal Care.

2.4. Protection against natural exposure to E. coli O157: H7

One hundred and ninety-two steers were blocked by weight, stratified by weight within block and assigned randomly to 24 pens on 11 May 2002. The cattle were housed in outdoor dirt-floored pens with a stocking density of 28 square meters of floor-space per animal. The finishing diet of 54.5% high moisture corn, 35% wet corn gluten feed, 5% corn silage, 2.5% alfalfa hay, and 5% supplement was used for all animals. The treatments (vaccination, yes or no) were randomly allocated to four pens within each of three weight lage, 2.5% alfalfa hay, and 5% supplement was used for all animals. The treatments (vaccination, yes or no) were randomly allocated to four pens within each of three weight blocks for a total of twelve repetitions per treatment. The treatments were initiated (treatment day 0) 9, 17, and 23 days after cattle arrived in the feedyard for blocks 1, 2, and 3, respectively. The vaccine was the same as described above, consisting of secreted proteins of E. coli O157:H7, and was administered subcutaneously (50 μg/dose) to cattle within assigned pens on treatment days 0, 21, and 42 of each block. Cattle within the control pens received an injection of adjuvant on the same schedule as vaccinated cattle.
Samples of rectal feces were collected for bacterial culture from cattle within each block on treatment days 0, 21, 42, 63, 84, and the day of marketing (treatment days 106, 104, and 105 of blocks 1, 2, and 3, respectively).

Outcome measures were pen-level performance and the proportion of animals per pen culture-positive for *E. coli* O157:H7. Feedlot performance and *E. coli* O157:H7 culture outcomes were analyzed after arcsine transformation of the proportions accounting for block as a random effect and repeated sampling for *E. coli* O157:H7.

Fecal samples were collected for *E. coli* O157:H7 as previously described [5] with modifications. Ten grams fecal samples were incubated 6 h in 90 ml Gram-negative (GN) broth containing 8 μg/ml vancomycin, 0.05 μg/ml cefixime, and 10 μg/ml cefsulodin. One ml of this culture was subjected to 157 immunomagnetic separation (Dynal, Lake Success, NY), and 20 μl of the bead-bacteria mixture was spread onto sorbitol-MacConkey plates containing cefixime (0.05 μg/ml) and potassium tellurite (2.5 μg/ml; CT-SMAC) and cultured overnight. Individual sorbitol-nonfermenting colonies were subcultured for isolation on CT-SMAC plates, and an individual sorbitol-nonfermenting colony from each plate was inoculated onto both MacConkey and Fluorocult (EM Science, Gibbstown, NJ) agars.

Isolates that fermented lactose but not sorbitol within 24 h and had a negative 4-methylumbelliferyl-β-D-glucuronide (MUG) reaction were streaked for isolation on blood agar and had a negative 4-methylumbelliferyl-β-D-glucuronide (MUG) reaction were streaked for isolation on blood agar were transferred into a 1.5 ml Eppendorf tube containing 50 μl sterile nuclease-free water and incubated at 100 °C for 6 min. The tube was then centrifuged at 2000 rpm for 2 min in a microfuge and the supernatant used as template. For each assay, template prepared from *E. coli* O157:H7 strain EDL933 (ATCC #43895) was used as a positive control, and one lacking any template was used as a negative control. The PCR was conducted in a 50 μl reaction mixture containing 2 μl template DNA, 10 mM Tris–HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 200 μM of each dNTP (Promega, Madison WI), and 1.5 U Taq DNA polymerase (Promega). Oligonucleotide primers (Table 1) were used at the following concentrations: 0.5 μM for those targeting the *eae*, *stx*₁, *stx*₂, and *rfbE*₀₁₅⁷;*H⁷* genes, and 0.2 μM for those targeting the *flIC*ᵢ₇ gene. Samples were amplified in a PTC-200 DNA engine (MJ Research Inc., Incline Village, NV) under the following cycling conditions. Conditions included an initial denaturation step of 5 min at 94 °C, followed by 34 cycles with denaturing at 94 °C for 45 s, annealing at 50 °C for 30 s, and extension at 72 °C for 90 s. The final cycle consisted of extension for 10 min at 72 °C, ramping to 50 °C at 0.1 °C/s, holding 50 °C for 5 min, ramping to 4 °C at 0.2 °C/s, and soaking at 4 °C. Samples were analyzed by standard agarose gel electrophoresis (10 μl per sample) on a 1.3% gel, stained with ethidium bromide, and then visualized under UV illumination. DNA molecular size standards (PCR marker, Promega, Madison WI) and positive and negative controls were included in every run.

### Table 1. Oligonucleotide primers used in multiplex PCR to confirm identity of isolates as *E. coli* O157:H7

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide sequence (5′–3′)</th>
<th>Product size (bp)</th>
<th>Target</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AE19</td>
<td>CAGGTCGTCGTGTCGCTGCTAAA</td>
<td>1087</td>
<td><em>eae</em></td>
<td>[33]</td>
</tr>
<tr>
<td>AE20</td>
<td>TCAGCGGTGGTGGATCAACCT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLICH-7F</td>
<td>GCGCTGTCGAGTTCTATCGAGC</td>
<td>625</td>
<td><em>flIC</em>₇</td>
<td>[34]</td>
</tr>
<tr>
<td>FLICH-7R</td>
<td>CAACGGTGACTTTATCGCCATTCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stx1F</td>
<td>TTCGCTTGCGAATAGGA</td>
<td>555</td>
<td><em>stx</em>₁</td>
<td>[35]</td>
</tr>
<tr>
<td>Stx1R</td>
<td>TTCCCCAGTTCAATGTAAGAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O157F</td>
<td>CGGACATCCATGTGATATGG</td>
<td>259</td>
<td><em>rfbE</em>₀₁₅⁷;<em>H⁷</em></td>
<td>[36]</td>
</tr>
<tr>
<td>O157R</td>
<td>TTGCCATGTGACGCTAATCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stx2F</td>
<td>GTGCCGTTTACTGAGTTTTCTTC</td>
<td>118</td>
<td><em>stx</em>₂</td>
<td>[35]</td>
</tr>
<tr>
<td>Stx2R</td>
<td>AGGGTCGATATCTCTGTGCTCC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3. Results

To test the vaccine potential of secreted proteins, supernatant proteins [containing type III secreted proteins (EspS and Tir)] were prepared from *E. coli* O157:H7, formulated at a dose of 100 μg/ml and used to vaccinate eight calves. A second group of calves which received a placebo vaccine as pre-immunization showed a 13-fold increase in specific antibody titre to type III secreted proteins after a single immunization, and after a booster vaccination on day 21, the EHEC vaccine group demonstrated a 45-fold increase in specific antibody titre while only one of the placebo vaccine group seroconverted (χ², P=0.0002). On each of the post-challenge days,
Table 2. Median serological response of yearling cattle to immunization with secreted proteins prepared from wild-type E. coli O157:H7 (EHEC), an isogenic tir mutant (ΔTir) or a placebo.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Anti-EHEC Day 0</th>
<th>Anti-EHEC Day 49</th>
<th>Anti-Tir Day 0</th>
<th>Anti-Tir Day 49</th>
<th>Anti-EspA Day 0</th>
<th>Anti-EspA Day 49</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EHEC</td>
<td>13</td>
<td>10 (10–100)</td>
<td>6400 (3200–12800)</td>
<td>100 (10–200)</td>
<td>1600 (800–3200)</td>
<td>100 (10–200)</td>
<td>400 (200–1600)</td>
</tr>
<tr>
<td>ΔTir</td>
<td>10</td>
<td>10 (10–100)</td>
<td>6400 (3200–25600)</td>
<td>10 (10–200)</td>
<td>200 (100–800)</td>
<td>100 (10–200)</td>
<td>300 (100–1600)</td>
</tr>
<tr>
<td>Placebo</td>
<td>25</td>
<td>10 (10–200)</td>
<td>10 (10–200)</td>
<td>100 (10–200)</td>
<td>200 (10–400)</td>
<td>100 (10–200)</td>
<td>100 (10–200)</td>
</tr>
</tbody>
</table>

Titres are expressed as median values of the last positive dilution of sera [31]. Numbers in parentheses represent the 25th–75th percentile.
ism for at least 1 day as compared to 15.4% of the EHEC (2 of 13, \( P=0.003 \)) and 30% (3 of 10, \( P=0.008 \)) of the ΔTir vaccinates.

To determine whether vaccination would have an effect on *E. coli* O157:H7 infection occurring from natural exposure, a clinical trial was conducted. Specifically, the trial evaluated the effect of vaccination on the proportion of feedlot steers shedding *E. coli* O157:H7 in their feces in typical feedlot conditions of exposure. Treatment groups did not differ in performance (viz. average daily gain, dry matter intake, gain to feed, marbling score, fat thickness, or yield grade).

The pre-treatment prevalence of animals shedding *E. coli* O157:H7 averaged 30%, and did not differ significantly between treatments (\( P=0.66 \)). The average proportion of cattle shedding *E. coli* O157:H7 differed (\( P=0.04 \)) over the five test-periods (treatment days 21, 42, 63, 84, and the day of marketing; 24, 10.9, 13.0, 5.7, and 21.4%, respectively); however, no interaction was observed between treatment and test-period (\( P=0.62 \)). The average proportion of cattle shedding *E. coli* O157:H7 in vaccine treated pens (8.8%) was significantly less (\( P=0.04 \)) than in non-vaccinated pens of cattle (21.3%) (Figure 4). A total of 362 *E. coli* O157:H7 isolates were obtained from the cattle in this clinical trial. By the five primer-pair multiplex PCR, all isolates were positive for the *rfbE* and *fliC* genes; 315 isolates were also positive for the *stx*2 and *eae* genes, but negative for *stx*1; 42 isolates were positive for all five genes.

4. Discussion

The data presented above demonstrate that virulence factors secreted by the type III system can be used as effective vaccine components for the reduction of colonization of cattle by *E. coli* O157:H7 in an experimental challenge model, and in a clinical trial testing under conditions of natural exposure within a feedlot setting. These proteins are major targets of the immune response in humans following infection [22], although calves do not usually mount a significant serological response against these proteins following natural exposure to the organism. However, cattle...
vaccinated with these proteins are primed and show an increase in anti-EHEC and anti-Tir titres following oral challenge with the organism [25].

Tir is likely required for colonization of the bovine intestine [25], and this is supported by the observation that a vaccine containing secreted proteins from a ΔTir *E. coli* O157: H7 strain was not as efficacious as an identical formulation from an isogenic wild-type isolate. However, the former vaccine was significantly more efficacious than a placebo suggesting that immunity against colonization is multifactorial in nature. This is supported by the Western blot analysis of the response to immunization in which several protein components as well as lipopolysaccharide were recognized.

The clinical trial was conducted under conditions of natural exposure in an environment typical of feedyards in the Central US and Canada. The prevalence of *E. coli* O157: H7 shedding observed among all of the cattle at the beginning of the trial and among control group cattle throughout the trial was typical of that previously observed in both research [32] and commercial feedyards [5]. During the trial, the prevalence of cattle shedding *E. coli* O157:H7 varied over time within both treatment groups.

Longitudinal studies have documented that within the same group of cattle the proportion of cattle shedding *E. coli* O157:H7 can vary greatly (1–80%) over the course of the feeding period [32]. In this study, even though prevalence of shedding varied by time period, vaccination resulted in proportionately less shedding in the five test-periods compared to controls. Three doses of vaccine were administered during the clinical trial; however, it is not clear that all three doses were necessary for effect. The prevalence of shedding was observed to decrease following the first vaccination in the clinical trial. Also, no interaction was observed between treatments and test-periods suggesting that the effect of vaccine on fecal shedding did not differ after the first vaccination. The issue is of practical importance since feedlot operators may be challenged to comply with the need to repeatedly vaccinate cattle, and greater numbers of doses increase the cost of feedlot cattle production. Therefore, the results of the first vaccine trial in calves described above suggests that a schedule of two immunizations would be sufficient to significantly reduce the numbers of animals shedding the organism.

The prevalence of non-O157 serotypes in North America appears to be increasing and represents a significant portion of EHEC infections in other geographical locations. Since the type III secreted antigens are relatively conserved among non-O157 EHEC serotypes, this vaccine formulation might be broadly cross-protective, in contrast to formulations based upon the specific O157 LPS antigen. The vaccine described here is relatively simple and economical to prepare, an essential requirement for any bovine vaccine. In addition, it emphasizes the feasibility of vaccinating an animal reservoir to potentially decrease human infections.
Acknowledgments

This research was supported by grants from the Beef Industry Development Fund, Alberta Agricultural Research Institute Strategic Emerging Issues Program, Canadian Bacterial Diseases Network Centre of Excellence, Bioniche Life Sciences, ID Biomedical, Alberta Research Council, Howard Hughes Medical Institute (HHMI), National Science and Engineering Research Council (NSERC), Canadian Institutes of Health Research (CIHR), United States Department of Agriculture (NRI-CGP 2000–02501 and 2001–02966) and the Nebraska Beef Council. BBF is a CIHR Distinguished Investigator, a HHMI International Scholar, and the UBC Peter Wall Distinguished Professor. We thank D. Wilson and the staff at VIDO and UNL for assistance with the vaccine trials. Published with the permission of the Director of VIDO. A contribution of the University of Nebraska Agricultural Research Division, Lincoln, NE 68583. Journal series no. 13929.

References


