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

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Abstract

Cattle are an important reservoir of *Escherichia coli* O157:H7 leading to contamination of food and water, and subsequent human disease. This pathogen colonizes its hosts by producing several proteins such as Tir and EspA that are secreted by a type III secretion system. These proteins play a role in colonization of the intestine, suggesting that they might be useful targets for the development of a vaccine to reduce levels of this organism in cattle. Vaccination of cattle with proteins secreted by *E. coli* O157:H7 significantly reduced the numbers of bacteria shed in feces, the numbers of animals that shed, and the duration of shedding in an experimental challenge model. Vaccination of cattle also significantly ($P = 0.04$) reduced the prevalence of *E. coli* O157:H7 in a clinical trial conducted in a typical feedlot setting. This strategy suggests it is possible to vaccinate cattle to decrease the level of *E. coli* O157:H7 shedding for the purpose of reducing the risk of human disease.
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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 10^8 CFU 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 [29,30] 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 3% 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

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

Primer	Oligonucleotide sequence (5′–3′)	Product size (bp)	Target	Reference
AE19	CAGGTCGTCGTGTCTGCTAAA	1087	*eae*	[33]
AE20	TCAGCGTGGTTGGATCAACCT			
FLICH-7F	GCGCTGTCGAGTTCTATCGAGC	625	*fliC*$_{h7}$	[34]
FLICH-7R	CAACGGTGACTTTATCGCCATTCC			
Stx1F	TTCGCTCTGCAATAGGTA	555	*stx*$_1$	[35]
Stx1R	TTCCCCAGTTCAATGTAAGAT			
O157F	CGGACATCCATGTGATATGG	259	*rfbE*$_{O157:H7}$	[36]
O157R	TTGCCTATGTACAGCTAATCC			
Stx2F	GTGCCTGTTACTGGGTTTTTCTTC	118	*stx*$_2$	[35]
Stx2R	AGGGGTCGATATCTCTGTCC			

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 cultured 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 O157 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 plates. Following overnight incubation, one colony per isolate from a blood agar plate was tested for *E. coli* O157 and H7 antigens by latex agglutination (Remel, Lenexa, KS).

Isolates that were positive for O157 antigen by latex agglutination (regardless of H7 results) were tested in a five primer-pair multiplex polymerase chain reaction (PCR) assay that detected genes for *E. coli* O157 (*rfbE*$_{O157:H7}$), H7 (*fliC*$_{h7}$), Shigatoxins 1 (*stx*$_1$) and 2 (*stx*$_2$), and intimin (*eae*). For PCR amplification, cells from an individual colony 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 MgCl$_2$, 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*$_{O157:H7}$ genes, and 0.2 μM for those targeting the *fliC*$_{h7}$ 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.

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 ug/ml and used to vaccinate eight calves. A second group of calves which received a placebo vaccine was also included. The group that received the EHEC vaccine 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 (χ^2, $P = 0.0002$). On each of the post-challenge days, fewer
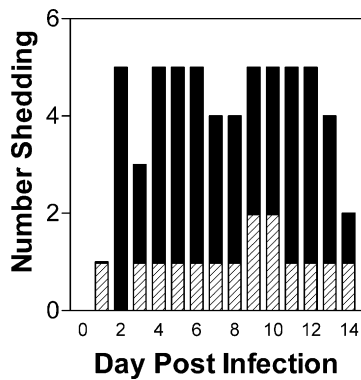
Fig. 1. Number of calves shedding *E. coli* O157:H7 on each day following experimental challenge. The strain used for infection [22–27] was grown to mid-exponential phase as described [22]. Bacteria were detected by direct plating of fecal samples that had been resuspended in saline on Sorbitol MacConkey agar supplemented with cefixime and tellurite [29]. Solid bars, placebo group; hatched bars, EHEC vaccine group.

EHEC-vaccinated animals shed bacteria compared to the placebo group (Fig. 1). Seven of eight placebo-immunized animals shed the bacteria during the trial and four of those animals shed for four or more consecutive days. Five of eight EHEC vaccine-immunized animals shed the bacteria at some point during the trial, but only one shed for more than two consecutive days, indicating that colonization was transient and significantly less than the placebo group. The total number of bacteria isolated from fecal samples was significantly lower among the EHEC-vaccinated group as compared to the placebo group (Wilcoxon Rank Sum Test, $P = 0.05$), with the former having a median of 6.25 colony forming units (CFU) per gram of feces recovered compared to a median value of 81.25 CFU/g for the latter.

No significant differences in anti-EHEC, anti-Tir or anti-EspA responses were observed between groups of six calves immunized with 50, 100, or 200 μg of supernatant, but all three were significantly higher than the placebo group on days 21 (boost) and 35 [25]. Thus, a second vaccine trial was conducted in which three groups of yearling cattle were immunized with supernatant prepared from the wild-type strain ($n = 13$), from a *tir* mutant [19] (ΔTir, $n = 10$) or a placebo ($n = 25$) on days 0, 21, and 35, followed by oral challenge with *E. coli* O157:H7 on day 49. The group that received the ΔTir vaccine showed a response of similar magnitude against total secreted proteins as the

group that received the vaccine prepared from the wild-type strain, but, as expected, a significantly reduced response to Tir (Wilcoxon Rank Sum Test, $P = 0.006$) (Table 2). However, the former group did show an increase in anti-Tir antibody levels (Wilcoxon Rank Sum Test, $P = 0.009$), indicating either exposure to an organism producing an immunologically-related molecule or natural exposure to *E. coli* O157:H7. This is further supported by the observation that there was an increase in the anti-Tir antibody titre in the placebo group on the day of challenge (Wilcoxon Rank Sum Test, $P = 0.002$) but no difference between the placebo or ΔTir groups ($P = 0.37$, Kruskal–Wallis ANOVA). The response to EspA, a type III secreted protein, was similar in both the EHEC and ΔTir vaccine groups ($P = 0.45$, Kruskal–Wallis ANOVA) and was significantly higher than the placebo-immunized animals ($P < 0.0001$).

The proteins secreted by the type III system were highly immunogenic in cattle (Fig. 2). EspB, EspD and Tir were all reactive and after the second immunization on day 21, a significant response against lipopolysaccharide was also observed. The kinetics of the immune response in a vaccinated animal (Fig. 2, bottom panels) show that anti-Tir antibodies were detectable following a single immunization, as were antibodies against 43 and 100 kDa proteins. The latter proteins were produced by the wild-type strain as well as the *sepB* and *tir* mutants and the 100 kDa protein is probably EspP, a non-type III EHEC secreted protein.

After oral challenge with *E. coli* O157:H7 on day 49, each group was monitored daily for fecal shedding of the organism for 14 days. In this experiment, bacteria were cultured after immunomagnetic enrichment [29,30] rather than direct plating as yearling cattle shed less than calves in this infection model. On the day of challenge, two animals in the placebo group were culture-positive for *E. coli* O157:H7 and were eliminated from the trial. The placebo-immunized animals shed the organism after challenge much more than those in the two EHEC vaccine groups (Fig. 3). Animals receiving the placebo shed the organism for a median of 4 days, significantly longer than the median of 0 day by the other two vaccine groups ($P = 0.0002$, Kruskal–Wallis ANOVA). Significantly fewer bacteria were recovered from the EHEC and ΔTir vaccine groups ($P = 0.04$, Kruskal–Wallis ANOVA). From day 2 post-infection onwards, 78.3% of the placebo animals (18 of 23 animals) shed the organism for at least 1 day as compared to 15.4% of the EHEC (2 of 13,

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

Group	N	Anti-EHEC		Anti-Tir		Anti-EspA	
		Day 0	Day 49	Day 0	Day 49	Day 0	Day 49
EHEC	13	10 (10–100)	6400 (3200–12800)	100 (10–200)	1600 (800–3200)	100 (10–200)	400 (200–1600)
ΔTir	10	10 (10–100)	6400 (3200–25600)	10 (10–200)	200 (100–800)	100 (10–200)	300 (100–1600)
Placebo	25	10 (10–200)	10 (10–200)	100 (10–200)	200 (10–400)	100 (10–200)	100 (10–200)

Titres are expressed as median values of the last positive dilution of sera [31]. Numbers in parentheses represent the 25th–75th percentile.
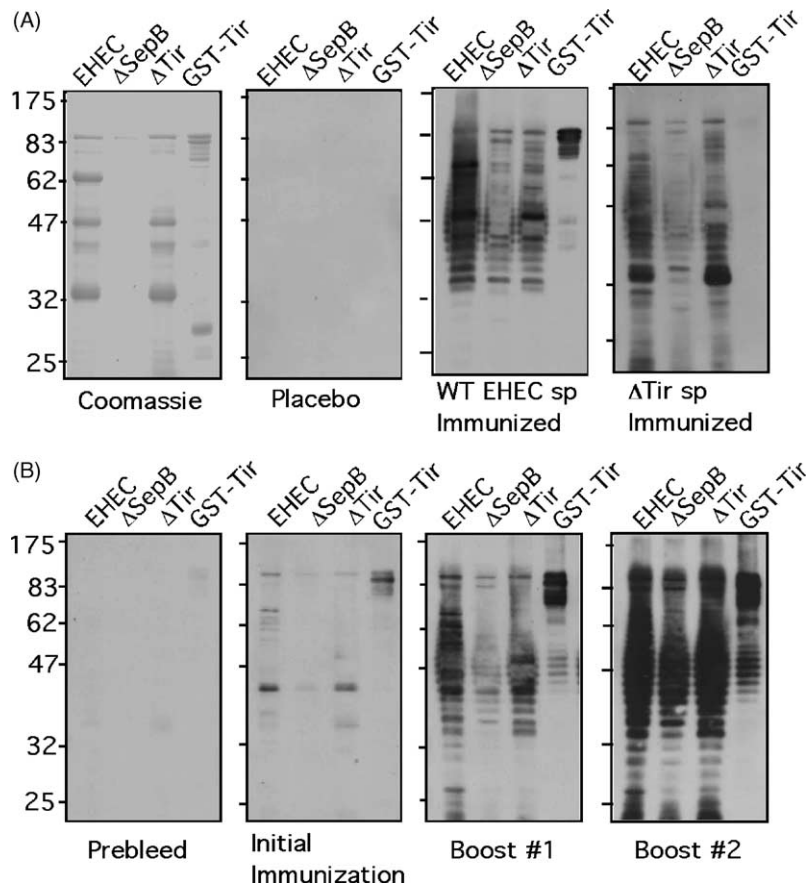
Fig. 2. Immunoblot analysis of sera from vaccinated yearling cattle against EHEC secreted proteins. Each blot contains secreted proteins from wild-type *E. coli* O157:H7 (EHEC), type III secretion mutant (ΔSepB), *tir* mutant (ΔTir) and a purified glutathione-s-transferase:Tir fusion protein (GST-Tir). Proteins were separated by SDS-10% PAGE and stained with Coomassie blue (A, upper left panel) or transferred to nitrocellulose and probed with representative sera from animals which received three immunizations with each vaccine formulation (A). The lower four panels (B) were probed with sera from one representative animal which received the EHEC vaccine, taken on days 0, 21, 35 and 49 of the trial (prebleed, after initial immunization, after boost #1, after boost #2, respectively).

$P = 0.003$) 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%) (Fig. 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*h7 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
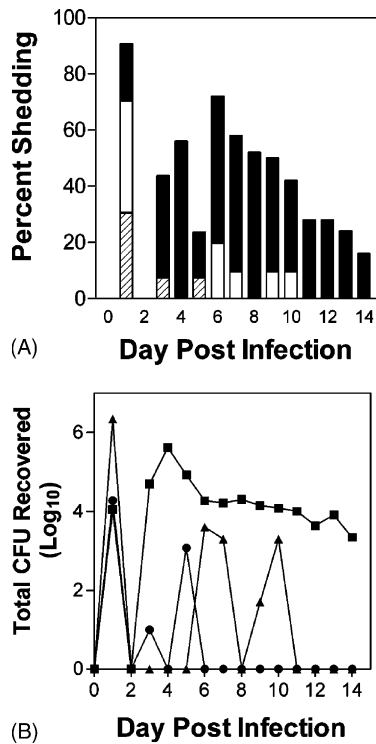
Fig. 3. Percentage of each group of yearling cattle shedding *E. coli* O157:H7 (A) and total number of bacteria recovered (B) on each day after experimental infection. Bacteria were detected in feces by plating on Sorbitol MacConkey agar supplemented with cefixime and tellurite following immunomagnetic enrichment as described [29]. (A) Solid bars, placebo; hatched bars, EHEC vaccine; open bars, ΔTir vaccine. (B) Squares, placebo group; circles, EHEC vaccine; triangles, ΔTir vaccine.

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*
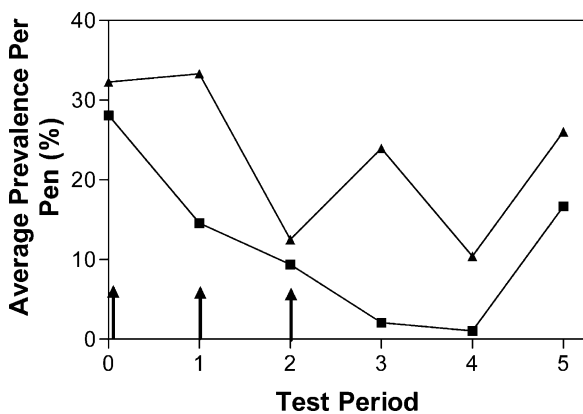

Fig. 4. The proportion of cattle shedding *E. coli* O157:H7 within pens of vaccinated and unvaccinated cattle in conditions of natural exposure. Arrows indicate the time of vaccination. Test-periods represent the day of sampling at 3 week intervals (treatment days 0, 21, 42, 63, 84, and the day of marketing), with 0 representing sampling prior to the first vaccination. Triangles, unvaccinated controls; squares, vaccinated animals.

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 most efficient number of vaccine doses must be tested in additional studies. However, 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.

Acknowledgements

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