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Immunological response of dairy cattle housed at an Ontario animal exhibit to vaccination with *Escherichia coli* O157 Type III secreted proteins

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Abstract

Interventions to reduce on-farm risk are important to help decrease the likelihood that the public will come into contact with *Escherichia coli* O157. Dairy cattle housed at an Ontario animal exhibit were immunized against *E. coli* O157, and their blood was tested to measure antibody titers to type III secretory proteins in the vaccine. Data collected during this retrospective study supports the conclusions that all animals, including young calves, immunologically responded to vaccination. At the end of a nine-month study period, those animals which received a two-dose vaccination protocol had similar antibody titers to the type III secretory proteins as those administered a three-dose protocol, and all animals immunologically responded to a single annual booster dose of vaccine.

Key words: dairy, *E. coli*, O157, vaccination

Résumé

Les interventions pour réduire le risque à la ferme sont importantes afin de diminuer les chances que le public rentre en contact avec *Escherichia coli* O157. Les bovins laitiers qui résidaient dans une exposition animale en Ontario étaient immunisés contre *E. coli* O157 et leur sang a été testé pour mesurer les titres d'anticorps aux protéines sécrétoires de type III du vaccin. Les données ramassées dans le cadre de cette étude rétrospective supportent la conclusion que tous les animaux, incluant les jeunes veaux, montraient une réponse immunitaire à la vaccination. Neuf mois après le début de l'étude, les animaux qui avaient reçu un protocole de vaccination à deux doses avaient des titres d'anticorps aux protéines sécrétoires de type III similaires à ceux des animaux qui avaient reçu un protocole à trois doses. Tous les animaux montraient une réponse immunitaire à la simple dose de rappel annuel du vaccin.

Introduction

Three-quarters of new and emerging human diseases are zoonotic.[21] Verocytotoxin-producing *Escherichia coli* (VTEC) are considered an important zoonotic pathogen. While many different VTEC serotypes exist, there are a small number which have been linked to human illness. Of these, the O157 serotype has been associated with 85 to 95% of the outbreaks and illnesses.[2,6] Human infection by *E. coli* O157 can lead to bloody diarrhea, haemolytic uremic syndrome, hemorrhagic colitis, kidney failure, and death. Approximately 75,000 infections, 2,100 hospitalizations, and 61 deaths are attributed to *E. coli* O157 annually in the United States (US).[11] The total cost of these infections in the US is estimated to be $993 million annually.[16]

E. coli O157 was first recognized as a cause of human illness in the early 1980s during an outbreak of severe bloody diarrhea traced to contaminated hamburgers.[6] This early linkage created the impression that infection caused by this pathogen could only be acquired by eating undercooked ground beef. An investigation of outbreaks between 1997 and 2001 indicated that 60% are attributed to the consumption of contaminated foods, and ground beef was the causative agent in 40.5% of the cases.[7] Based on a review of data between 2003 and 2007, the consumption of contaminated produce was found to be the cause in 54% of human *E. coli* O157 illnesses.[16] This knowledge has increased our understanding that multiple interventions are needed to reduce the risk of infection.[9]

Human illnesses due to *E. coli* O157 have been linked to sources other than consumption of food prod-

ucts, including ingestion of contaminated water and direct contact with animals and their environment. Despite awareness of the potential for human disease, there continues to be reports of *E. coli* O157 illnesses associated with animal exhibits, which account for 4% of all outbreaks.[15] The increasing numbers of reported outbreaks may be related to the growing number of urban people who lack antibodies gained from field exposure in a rural setting.[5] With approximately six million people attending animal exhibits in the US annually, this represents a significant public health risk.[8]

Although *E. coli* O157:H7 has been found in, on and around ruminants including cattle, deer, goats, and sheep, cattle are considered to be the primary reservoir.[6] Cattle remain asymptomatic[12] but shed the bacteria in their feces. Manure and run-off are sources of contamination from the animal reservoir to the human population. On-farm prevalence of *E. coli* O157 varies greatly depending on the environmental conditions, type of farm and season,[13] and the majority of farms will have positive animals at some point in time.[6]

Several interventions are being explored to decrease VTEC bacterial load in the farm environment before animals go to slaughter or exhibits. One practical intervention strategy is to prevent colonization of the gut. Colonization of cattle by *E. coli* O157 requires type III secreted proteins (TTSP),[3] including Translocated Intimin Receptor (Tir) and *E. coli* secreted Protein (Esp) A, all of which are important for attachment of the bacteria to host cells.[14]

Several field and controlled challenge studies have demonstrated that vaccination against components of the type III system causes a reduction of the duration and magnitude of shedding, lower colorectal colonization, reduced hide contamination, and a reduction in environmental prevalence and transmission.[13,14,18,19]

The relationship between antibody titers, protection against *E. coli* colonization, and shedding have been evaluated in controlled challenge settings as well as natural exposure field studies. Serological responses to the proteins secreted by the type III system are highly immunogenic in cattle, and have been associated with a significant decrease in shedding and colonization in studies conducted under controlled challenge settings or in clinical trials under natural exposure to *E. coli* O157.[1,10,13,14]

Allen[1] investigated the efficacy of a type III secretory protein vaccine in a controlled challenge model. He reported a robust serological response to vaccination corresponding to a significant reduction in the number of animals shedding *E. coli* O157 and the number of organisms shed per animal. Allen concluded that vaccination was protective and had a significant ability to decrease the number of bacteria being shed into the environment, thereby reducing environmental and hide contamination.

Implementing the use of on-farm interventions, such as vaccination, against this important human pathogen could significantly reduce the number of human illnesses.[20] This is particularly relevant on this exhibit farm where direct contact between cattle and children could lead to illnesses.

This retrospective cohort study was conducted to answer questions about exhibit animal use of a vaccine against *E. coli* O157. Specifically, the objectives of this study were to: 1) determine the antibody response to vaccination in previously immunized mature cows and naïve calves, 2) compare the difference in antibody titers at the end of the study period in mature cows following application of a two- or a three-dose vaccination protocol, and 3) measure the antibody response in cattle following an annual booster.

Materials and Methods

Animals

Eighty dairy cattle (lactating and dry) and twelve calves, belonging to the Canada Agriculture Museum in Ottawa, Canada, were immunized against *E. coli* O157 TTSP over a four-year time period from 2007 to 2010, and blood was drawn during 2009 and 2010 (the study period).

Naïve calves, two to eight months of age, received four doses of vaccine and blood was collected three times during the study period (Table 1a). During the nine-month study period, any calves under four months of age received a half dose (1 mL) of vaccine. Mature cows (age one to 12 years) were vaccinated against *E. coli* O157 up to five times during the two years prior to the study period (Table 1b). Cows received either two or three doses of vaccine and blood samples were taken at different periods. No adverse events, as defined by anaphylactic shock, were reported during the study period.

Description of Vaccine

Supernatant proteins from *E. coli* O157 were produced and combined with adjuvant as described previously.[1] The vaccine contains type III secretory proteins including Tir, EspA, and total Enterohemorrhagic *E. coli* (EHEC). The antigen used in this trial was the same antigen used to obtain a commercial license in Canada.[a] Two milliliters of the *E. coli* O157 TTSP vaccine was administered subcutaneously.

Determination of Serum Response

Serum antibody titers to each of *E. coli* O157 antigens was determined by enzyme-linked immunosorbent assay (ELISA). Enterohemorrhagic *E. coli* protein extract, EspA, and Tir purified antigens were prepared by Bioniche Life Sciences Inc.[b] Each antigen

Table 1. Schedule of vaccine administration and blood sample collection for naïve calves and cows administered two and three doses during the study period (shade area) and following an annual booster.

(a) Naïve calves (N=12)

Day of study	Vaccination	Blood sample
0		X
12	X	
27	X	X
178	X	
218	X	
241		X

(b) Cows (N=80)

Study period					
Two doses			Three doses		
Day of study	**Vaccination**	**Blood sample**	**Day of study**	**Vaccination**	**Blood sample**
0		X	0		X
12	X		12	X	
27			27	X	X
178	X		178	X	
207		X	207		X
Period following annual booster					
Day of study	**Vaccination**	**Blood sample**	**Day of study**	**Vaccination**	**Blood sample**
572	X	X	572	X	X
614		X	614		X

was diluted with carbonate-bicarbonate coating buffer (1.59 g Na_2CO_3 per liter and 2.93 g $NaHCO_3$ per liter, pH 9.6) to a concentration of 0.1 µg/100 µl. To each well of a 96 well ELISA plate,[c] a volume of 100 µl of one antigen was applied. Each ELISA plate was incubated overnight at 39.2°F (4°C) with each well in carbonate-bicarbonate coating buffer. The wells were washed three times using 0.05% PBS-Tween 20 (PBST) and were then blocked for one hour at room temperature using 0.1M Tris-HCl containing 0.14M NaCl and 0.05% Tween 20 plus 1% w/v skim milk powder (TBST+SM). Sera were pre-diluted in TBST+SM to 1/40, and then 1:4 serial dilutions were made. A negative (TBS+SM) and a positive control (pooled serum samples from previous studies) were on each plate.

Each sample was analyzed in duplicate. To measure total IgG antibody titer, 100 µl of goat anti-bovine IgG alkaline phosphatase labelled antibody[d] diluted 1/2500 in TBST+SM was added to each well, washed six times, and incubated for one hour at room temperature (73.4±3.6°F; 23±2°C). After washing, 100 µl of PNPP substrate[e] was added to each well and incubated for two hours at room temperature. Plates were stopped using a solution containing 100 mL PBST, 111.6 g EDTA disodium salt (BDH ACS-345), 13g NaOH with a pH adjusted to 8.0 and read at 405 nm in a microplate reader.[f]

All absorbencies at 405 nm were subtracted from the blank (TBST+SM). The end-point titer of each serum sample was the dilution that gave an A405 nm double the A405 nm of the negative control. Microsoft Excel's[g] forecast function was used to determine an "intercept" titer at the cut-off OD (0.05).

Statistics

The statistical model included effects of day of blood sample relative to vaccination or a two or three dose vaccination protocol, which were individually regressed on the dependent variables: antibody titers of EHEC, EspA or Tir. Data were analyzed by one-way analysis of variance.

All variables were tested for normality and homogeneity of variance, and were found to fit both criteria. Data were considered significantly different at $P < 0.05$.

Results

Antibody Response in Previously Immunized Cows

In previously immunized cows (n=64), the blood collection prior to first vaccination on day 0 was eight months after their previous immunizations. Mean antibody titers at that collection time were 1,543, 1,009, and 1,058 for EHEC, EspA, and Tir, respectively. Compared to pre-vaccination titers, there was a significant increase ($P < 0.05$) in antibody titers to EHEC, EspA, and Tir on day 27 in response to the vaccination (Figure 1). All animals serologically responded to vaccination.

Several animals received five vaccinations (n=28) over the two years prior to the study period, while others received only a single vaccination (n=17). The number of previous vaccinations did not affect antibody titers measured prior to, or on antibody titer responses after, the first vaccination during the study period ($P \geq 0.05$).

Antibody Response Following Application of a Two- or Three-Dose Vaccination Protocol

During the study period, 21 cows received two doses of vaccine and 31 cows received three doses of vaccine. There were no statistically different levels of antibody titer against EHEC, EspA, and Tir ($P \geq 0.05$) observed on day 207 between the two- and three-dose groups (Figure 2).

Antibody Response in Naïve Calves

While naïve calves had pre-existing antibody titers to EHEC, Tir, and EspA prior to vaccination, measurement of antibodies confirmed all calves responded to vaccination.

Naïve calves (n=12) showed an immune response to Tir and EspA ($P < 0.05$) on day 27 after the sensitizing dose, whereas there was no significant increase ($P \geq 0.05$) in EHEC titers (Figure 3). On day 241, after the administration of the fourth dose of vaccine, antibody titers for EHEC, EspA, and Tir were higher than antibody titers recorded before the sensitizing dose ($P < 0.05$; Figure 3). Titers for EHEC and Tir ($P < 0.05$), but not for EspA ($P \geq 0.05$), were higher after the fourth than after the first dose of vaccine.

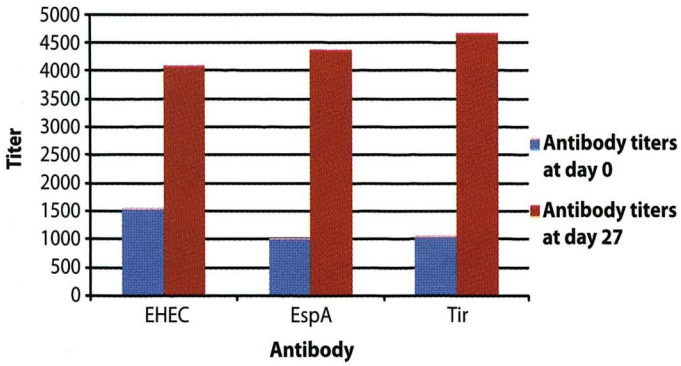

Figure 1. Antibody response in previously immunized cows. The cut-off optical density (OD) used was 0.05. Each antibody titer was significantly greater at day 27 after vaccination compared to the titers recorded in blood samples taken immediately prior to vaccination ($P < 0.001$ for all titers).

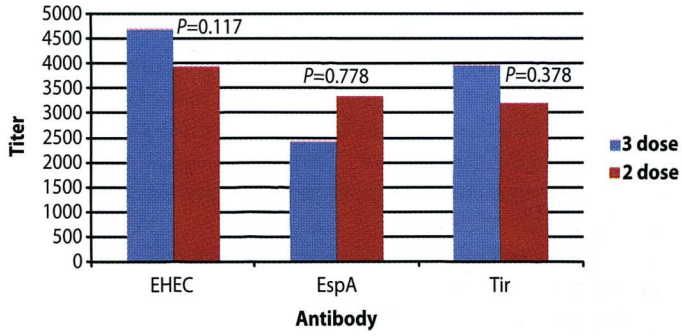

Figure 2. Antibody titers on day 207 to EHEC, EspA, and Tir in cows receiving either a 2- or 3-dose vaccination protocol. The cut-off optical density (OD) used was 0.05. At day 207 after final vaccination there was no difference in the titers of EHEC ($P = 0.117$), EspA ($P = 0.778$), and Tir ($P = 0.378$) in cows which were administered two (n=21) or three (n=31) doses of vaccine.

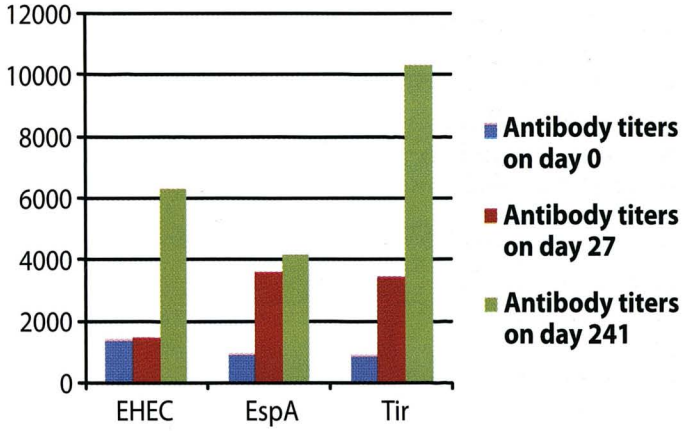

Figure 3. Antibody response in naïve calves. The cut-off optical density (OD) used was 0.05. At day 27, calves (n=12) showed an immune response to Tir ($P = 0.037$) and EspA ($P = 0.031$), and not EHEC ($P = 0.886$) titers. On day 241 EHEC ($P < 0.001$) and Tir ($P = 0.003$) titers showed a significant increase, while EspA ($P = 0.778$) did not.

A subset analysis of the six naïve animals under four months of age receiving only 1 mL (half dose) of vaccine was undertaken. A significant increase in antibody titers to Tir and EHEC ($P < 0.05$) was observed in these calves; however, the increase in EspA titer after the series of three doses was not significant (data not shown).

Antibody Response to Annual Booster

Antibodies were measured in 43 cows before vaccination (day 572) and six weeks after the annual booster (day 614) to determine if there was an antibody titer increase. Results showed a significant increase in antibody response to Tir, EspA, and EHEC ($P < 0.05$; Figure 4).

Supplementary Dairy Cow Data

Further investigation of antibody titers was undertaken in a small field study, where naïve dairy cattle (n=10) not previously vaccinated were found to have a low level of antibody titers. These cows were vaccinated with TTSP against *E. coli* O157 on days 0, 21, and 51, and antibody titers were compared to those of non-vaccinated cattle. Significant increases to Tir, EspA, and EHEC antibodies were measured on days 31 (after two doses of vaccine; $P < 0.05$) and 65 (after three doses of vaccine; $P < 0.05$). EHEC and Tir antibody responses increased between 40- and 60-fold, while EspA antibody responses increased 21-fold (Figure 5), confirming the immunological response seen by Potter *et al*[14] and Allen *et al*[1] in seronegative calves under controlled challenge.

Discussion

The purposes of this retrospective study were: 1) to determine the serological response of mature and naïve animals to vaccination with TTSP antigens in a sub-unit vaccine against *E. coli* O157 under field conditions, 2) to compare the difference in immune response of animals to a two- or three-dose vaccination protocol, and 3) to measure antibody response following annual booster.

Experimental studies have demonstrated that vaccinated cattle mount a significant immune response following oral challenge with *E. coli* O157.[13,14,18,19] In practice, the entire herd is routinely inoculated when a vaccination protocol is implemented, creating a herd immunity effect which correspondingly reduces the degree of pathogen shedding.[13] Reduced pathogen shedding coupled with heightened resistance increases the likelihood of herd immunity and disease avoidance. Serologic studies have shown that by the time of weaning the majority of cattle have been exposed to *E. coli* O157,[6] and research suggests that shedding by cattle will increase during periods of stress and changing environmental conditions.[17]

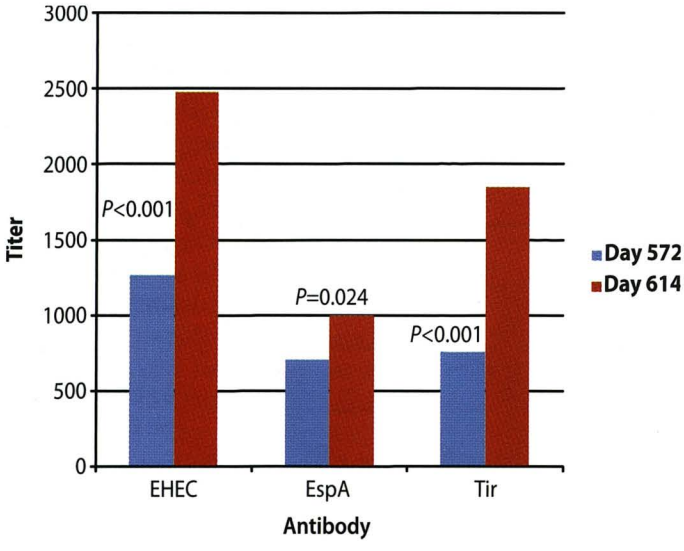

Figure 4. Antibody response to annual booster. The cut-off optical density (OD) used was 0.05. The titers to EHEC, EspA, and Tir were recorded in cows (n=43) in response to an annual revaccination protocol. Titers to EHEC, EspA, and Tir ($P < 0.001$, $P = 0.024$, $P < 0.001$, respectively) were elevated six weeks after revaccination.

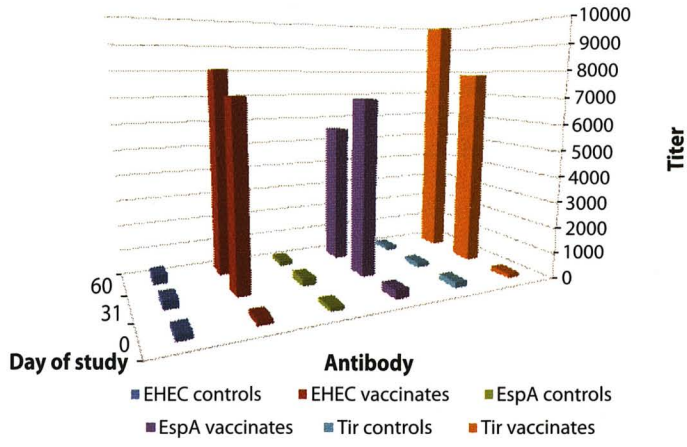

Figure 5. Supplemental dairy cow data. The cut-off optical density (OD) used was 0.05. The immunological response of seronegative calves (n=10) to vaccination at day 0, day 21, and day 51 was compared to calves not vaccinated. The titers of EHEC, EspA, and Tir at the time immediately before first vaccination were not different between groups. At day 31 (10 days after the second vaccination) and at day 60 (nine days after the third vaccination), titers to EHEC, EspA, and Tir were elevated ($P < 0.001$) compared to time-matched control animals.

The vaccine against *E. coli* O157 elicits protection through immunological response to key protective TTSP; Tir and EspA. The rectal-anal junction is considered the primary site of colonization.[12,13,18] Immunization of cattle with these key antigens stimulates resistance to colonization and reduces shedding.[13] Peterson *et al*[13] reported vaccinated cattle produced antibodies against EHEC, Tir, and EspA. Vaccinated animals were 59% less likely to shed the bacteria,[13] and were 98.3% less likely to be colonized.[12] Potter *et al*[14] and Allen *et al*[1] correlated increased antibody titers with reduced shedding of the organism. An objective of immunization is to establish long-lived Memory B cells. Once an animal has been successfully sensitized through vaccination, Memory B cells allow the animal to respond quickly to future exposures.[4] Data from this study successfully demonstrated an immunologic response shortly after vaccination and upon annual revaccination.

In a controlled challenge model utilizing seronegative cattle,[14] there was a 13-fold increase in antibody titers to TTSP after a single vaccination, and a 45-fold increase at 21 days after booster vaccination. Other researchers measured biological relevance in a field challenge using cattle with pre-vaccination titers as a four-fold minimum increase in antibodies and found that 84 days after the final vaccination there was no significant difference in EspA, EspB, Tir and Intimin when compared to day 0 of the study.[13] In a previous study, maximum antibody titer response was seen 14 days after vaccination.[1] In this study, the naïve animals had a four-fold increase in titers to EspA and Tir a month (day 27) after their first vaccination. On day 241, after their final vaccination, they had a five-fold increase in titers to EHEC and EspA, and a twelve-fold increase to Tir. Similarly, cows showed a four-fold increase a month (day 27) after their first vaccination and a 3.5-fold increase on day 207, after the last vaccination. All responses were biologically relevant in animals receiving either a two- or three-dose vaccination protocol, regardless of previous vaccination status.

Data from the present study likewise revealed no significant difference in antibody titer response in cattle that received three doses of vaccine compared to cattle that received two doses of vaccine. Data also established all animals demonstrated a significant increase in antibody titers upon vaccination, including naïve calves under four months of age and upon annual booster.

Conclusion

Veterinary medicine plays a key role in protecting public health from zoonotic agents such as *E. coli* O157. This study demonstrated that all animals, including young calves, immunologically responded to vaccination against *E. coli* O157 TTSP. A two-dose vaccination schedule provided a similar level of antibody titer as the three-dose protocol, and all animals immunologically responded to a single annual booster vaccination.

Endnotes

[a]Econiche™ (Bioniche Life Sciences, Belleville, Ontario, Canada) is approved in Canada for vaccination of healthy cattle as an aid in the reduction of shedding of *E. coli* O157.
[b]Bioniche Life Sciences, Belleville, Ontario, Canada
[c]Thermo Scientific/Nunc, Rochester, NY
[d]KPL, Inc., Gaithersburg, MD
[e]Sigma–Aldrich, Oakville, ON
[f]BioTek PowerWave; Fisher Scientific, Ottawa, Ontario, Canada
[g]Microsoft Excel, One Microsoft Way, Redmond, WA

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