Antibodies Directed against Shiga-Toxin Producing *Escherichia coli* Serotype O103 Type III Secreted Proteins Block Adherence of Heterologous STEC Serotypes to HEp-2 Cells

Taseen S. Desin[¤]*, Hugh G. Townsend, Andrew A. Potter

Vaccine & Infectious Disease Organization–International Vaccine Center, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

¤ Current address: Basic Sciences Department, College of Science & Health Professions, King Saud bin Abdulaziz University for Health Sciences, Riyadh, Saudi Arabia
* desint@ksau-hs.edu.sa

Abstract

Shiga toxin-producing *Escherichia coli* (STEC) serotype O103 is a zoonotic pathogen that is capable of causing hemorrhagic colitis and hemolytic uremic syndrome (HUS) in humans. The main animal reservoir for STEC is ruminants and hence reducing the levels of this pathogen in cattle could ultimately lower the risk of STEC infection in humans. During the process of infection, $STEC_{O103}$ uses a Type III Secretion System (T3SS) to secrete effector proteins (T3SPs) that result in the formation of attaching and effacing (A/E) lesions. Vaccination of cattle with STEC serotype O157 T3SPs has previously been shown to be effective in reducing shedding of $STEC_{O157}$ in a serotype-specific manner. In this study, we tested the ability of rabbit polyclonal sera against individual $STEC_{O103}$ T3SPs to block adherence of the organism to HEp-2 cells. Our results demonstrate that pooled sera against EspA, EspB, EspF, NleA and Tir significantly lowered the adherence of $STEC_{O103}$ relative to pre-immune sera. Likewise, pooled anti-$STEC_{O103}$ sera were also able to block adherence by $STEC_{O157}$. Vaccination of mice with $STEC_{O103}$ recombinant proteins induced strong IgG antibody responses against EspA, EspB, NleA and Tir but not against EspF. However, the vaccine did not affect fecal shedding of $STEC_{O103}$ compared to the PBS vaccinated group over the duration of the experiment. Cross reactivity studies using sera against $STEC_{O103}$ recombinant proteins revealed a high degree of cross reactivity with $STEC_{O26}$ and $STEC_{O111}$ proteins implying that sera against $STEC_{O103}$ proteins could potentially provide neutralization of attachment to epithelial cells by heterologous STEC serotypes.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is an enteric pathogen that causes diarroheal illness in humans which can lead to hemorrhagic colitis and haemolytic uremic syndrome (HUS), one of the main causes of renal failure in children [1]. Shiga toxins produced by this pathogen play an important role in causing these clinical manifestations. Currently, there is no treatment available for human STEC infections other than supportive care as the administration of antibiotics can exacerbate the disease. STEC O157:H7 is the predominant serotype associated with human infections in North America, while non-O157:H7 serotypes such as O103, O26, O111 are more prevalent in many European countries, South America and parts of Australia [1,2,3] The main reservoir for STEC is ruminants [4] and therefore intervention strategies aimed at lowering the levels of this pathogen in cattle could ultimately result in improved human health [5].

During the process of infection, STEC uses a Type Three Secretion System (T3SS) to inject virulence factors known as effector proteins directly into host cells, leading to the formation of attaching and effacing lesions (A/E) lesions, which are hallmarks of STEC infections. The major structural components of the STEC T3SS include EspA (filament), EspB and EspD (translocon complex) [6]. The STEC T3SS secretes over 50 effector proteins that are encoded on the LEE Pathogenicity Island or elsewhere on the chromosome (non-LEE effectors) [7]. The translocated intimin receptor, Tir, is an effector protein which enters host cells and forms a receptor that binds to intimin that is expressed on the surface of STEC cells [6]. Many studies have shown that the STEC T3SS is essential for colonization of cattle, implying that this is a major virulence factor employed by this pathogen [8,9,10].

Vaccination of cattle with STEC$_{O157}$ T3SP's has shown to be an effective strategy in reducing the shedding of STEC$_{O157}$ [11,12,13,14,15,16,17,18,19]. However, this protection appears to be serotype specific [20,21]. Therefore, alternative antigens need to be identified that offer protection against non-O157 STEC serotypes. Recently, it has been shown that anti-sera to an extract of STEC$_{O157}$ T3SPs had the highest degree of cross-reactivity with STEC$_{O103}$ recombinant T3SPs [20], suggesting that STEC$_{O103}$ T3SPs may have cross-protective potential. In this study, we tested the effect of sera against STEC$_{O103}$ recombinant proteins on STEC$_{O103}$ and STEC$_{O157}$ adherence to HEp-2 cells. Moreover, we tested the vaccine potential of the recombinant proteins against STEC$_{O103}$ challenge in mice.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains used in this study comprised of *E. coli* N01-2454 (O103:H2), EDL933 (O157:H7), CL9 (O26:H11) and CL101 (O111:NM) [22,23]. For cloning and protein expression, we used the *E. coli* K-12 lab strains, JM109 (*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r$_k^-$, m$_k^+$), *rel*A1, *sup*E44, Δ (*lac-pro*AB), [F′ *tra*D36, *pro*AB, *laq*I^{q}ZΔM15]) and BL21 (F$^-$, *dcm*, *ompT*, *hsdS$_B$* (r$_B^-$, m$_B^-$), *gal*, λ(DE3)) obtained from Qiagen and Invitrogen, respectively. The strains were grown in Luria Bertani (LB) medium at 37°C in an orbital shaker (250 rpm), unless otherwise stated. *E. coli* serotypes O103 and O157 were transformed with a green fluorescent protein expressing plasmid, pNR78, for visualization by flouresence microscopy during the adherence inhibition assays as described [21]. Plasmid pNR78 was constructed in our lab by amplifying the GFP gene from pQBI-25 (Quantum Biotechnologies) which was cloned downstream of the GroEL promoter.

Protein expression and purification

The STEC serotype O103:H2 T3SS genes *escC*, *espA*, *espB*, *espF*, *espG*, *espR1*, *nleA*, *nleE*, *nleF*, *nleG2*, *nleH*, *sepD*, *tccp2* and *tir* were amplified by PCR (Applied Biosystems) based on the

sequence provided by GenBank[®]. Similarly, for cross reactivity studies, *espA*, *espB*, *espF*, *nleA* and *tir* from STEC serotypes O26 and O111 were amplified by PCR. The genes were cloned in either pQE-30 (Qiagen), pET-15b (Novagen), pGEX-5X-1 (GE Healthcare) or pGEX-5X-3, of which the first two are 6x His-tagged protein expression vectors while the latter are Glutathione S-transferase (GST)-fusion expression vectors. The constructs were confirmed by PCR and sequencing (Plant Biotechnology Institute, Saskatoon). Proteins were expressed in *Escherichia coli* K-12 lab strains (JM109 or BL21) and purified using either the method described in the QIAexpressionist™ manual (Qiagen) for His-tagged proteins or the GST Gene Fusion System Handbook (GE Healthcare) for the GST-fusion protein. Purified protein samples were greater than 90% pure as determined by SDS-PAGE followed by Coomassie blue staining as described previously [20].

Raising polyclonal anti-sera to STEC$_{O103}$ T3SS recombinant proteins

Purified recombinant proteins (100 μg each) were formulated with 30% Emulsigen D (MVP Laboratories) and two New Zealand White rabbits (Charles River) per STEC$_{O103}$ recombinant protein were immunized subcutaneously on day 0, followed by booster injections on days 21 and 42. The rabbits were euthanized on day 56 and sera were collected. Antibody titers against STEC$_{O103}$ recombinant proteins were confirmed using ELISA in duplicate wells as previously described [20]. For antibody titer determinations, the cut-off value was considered to be the average of the blank and two standard deviations. All rabbits used in this study were handled and treated in accordance with the guidelines provided by the Canadian Council on Animal Care (CCAC) as administered by the University Committee on Animal Care and Supply (UCACS), protocol 1994–213. This protocol was approved by the UCACS at the University of Saskatchewan for the present study.

Cell culture

HEp-2 cells (ATCC[®] CCL-23™, CEDARLANE[®]) were grown in HyClone Dulbecco modified Eagle medium (DMEM; Thermo Scientific) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories) and 1% HEPES Buffer (Invitrogen) at 37°C in a 5% CO_2 incubator. One day prior to the adherence inhibition assays, 10^5 cells per well were seeded onto eight well chamber slides (Nunc) and allowed to incubate overnight.

Adherence inhibition assays

Adherence of STEC$_{O103}$ and STEC$_{O157}$ to HEp-2 cells was assessed using an assay described elsewhere [21]. Briefly, an overnight culture of STEC grown in LB media was subcultured (1:100) into DMEM containing 10% FBS and 1% HEPES Buffer for 2 hours (until the OD$_{600}$ was 0.2) at 37°C and 5% CO_2 without shaking. For testing the effect of pooled sera against STEC$_{O103}$ T3SPs on adherence, HEp-2 cells were infected with 25 μl of STEC (1.7×10^6 colony forming units), 10 μl of each serum and 225 μl fresh DMEM. The effect of individual anti-serum was tested by infecting HEp-2 cells with 25 μl of STEC (1.7×10^6 CFU), 20 μl of anti-serum and 225 μl fresh DMEM (anti-O103 antibodies were prepared as described previously [21]). The chamber slides were incubated at 37°C and 5% CO_2 for 3 hours (STEC$_{O157}$) or 3.5 hours (STEC$_{O103}$). The slides were washed six times with 200 μl Phosphate Buffered Saline (PBS, 0.1M pH 7.2) and fixed with 200 μl PBS containing 3.7% Formaldehyde. This was followed by two washes with PBS after which the slides were allowed to air dry. Coverslips were mounted with Vectashield[®] (Vector) containing DAPI and sealed. The slides were visualized under the fluorescent microscope (Axiovert 200 inverted microscope–Zeiss). Bacteria were observed under FITC, while HEp-2 cells were observed under DAPI. Each experimental group

was first tested using 2 replicate wells in an 8 well chamber slide and 4 random grids were examined per well under the fluorescent microscope as described below. After observing clear differences in STEC adherence to HEp-2 cells between the different treatments, the experiments were repeated independently on a separate occasion using 8 replicates per test group as previously published [21] with 4 random grids per well used for enumerating the number of STEC per HEp-2 cell. The resulting pictures (4 under FITC and 4 under DAPI) per well (total of 8 pictures per well) were used to enumerate the number of STEC and HEp-2 cells per well. The total numbers of STEC per grid were then divided by the total numbers of HEp-2 cells per grid to determine the number of STEC per HEp-2 cell in one grid. This was repeated for the 8 duplicate wells per group, resulting in a total of 64 pictures per test group. Each data point in Figs 1 and 2 represent the average number of STEC per HEp-2 cell from 4 counts (4 random grids) per well. For statistical analysis, the median STEC per HEp-2 cell across the different test groups were compared using a non-parametric analysis as described below.

Immunization of mice with $STEC_{O103}$ T3SPs

Twenty four BALB/C mice were obtained from Charles River Canada. Mice were housed at the VIDO Animal Care Facility (University of Saskatchewan) and handled in accordance with the guidelines provided by the CCAC as administered by the UCACS, protocol 1998–0003. This protocol was approved by the UCACS at the University of Saskatchewan for the present study. Mice were randomly divided into two groups with 12 mice per group. The mice were immunized subcutaneously on Day 0 with 100 µl of either PBS (0.1M, pH 7.2) or a pool of $STEC_{O103}$ recombinant proteins EspA, EspB, EspF, NleA and Tir (1 µg of each protein) followed by a second immunization at Day 21. The vaccines were formulated with 30% Emulsigen D (MVP Laboratories). Sera were collected prior to both immunizations on day 0 and day 21 as well as prior to challenge with $STEC_{O103}$ on day 35. Antibody titers were determined using ELISA in duplicate wells as described previously [20]. For antibody titer determinations, the cut-off value was considered to be the average of the blank and two standard deviations. The mice were challenged as described below.

STEC mouse colonization model

For colonization of mice, we used the streptomycin-treated model as previously described [24,25]. Briefly, mice were given water containing Streptomycin Sulfate (5 g/L) on day 32 for two days. Subsequently, mice were deprived of food and water for 24 hours. On Day 35, mice were orally challenged with 100 µl of 10^9 cfu of $STEC_{O103}$ Nal^r (resuspended in 20% sucrose). The mice were permitted access to food and water containing Streptomycin for the rest of the experiment. Fecal pellets were collected every 3 days for 21 days post challenge. Shedding of $STEC_{O103}$ was monitored by incubating the fecal samples in 1 ml LB broth for 2 hours at room temperature to allow the pellets to soften. The samples were vortexed, serially diluted in PBS and plated on MacConkey Sorbitol Agar containing Nalidixic Acid (15 µg/ml), Cefixime (5 µg/ml) and Potassium Tellurite (2.5 µg/ml). The plates were incubated overnight at 37°C and STEC colonies were enumerated the following day. Bacterial counts were expressed as cfu per gram of fecal content.

Cross-reactivity of $STEC_{O103}$ T3SS recombinant protein specific sera

Purified $STEC_{026}$ and $STEC_{0111}$ EspA, EspB, EspF, NleA and Tir recombinant proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane using a Mini Trans-Blot Electrophoretic Cell (Bio-Rad) as per the manufacturer's instructions. The membranes were probed with either polyclonal sera (1:2500) from mice vaccinated with a pool of the corresponding $STEC_{O103}$ recombinant proteins or with rabbit polyclonal sera (1:2500) raised against
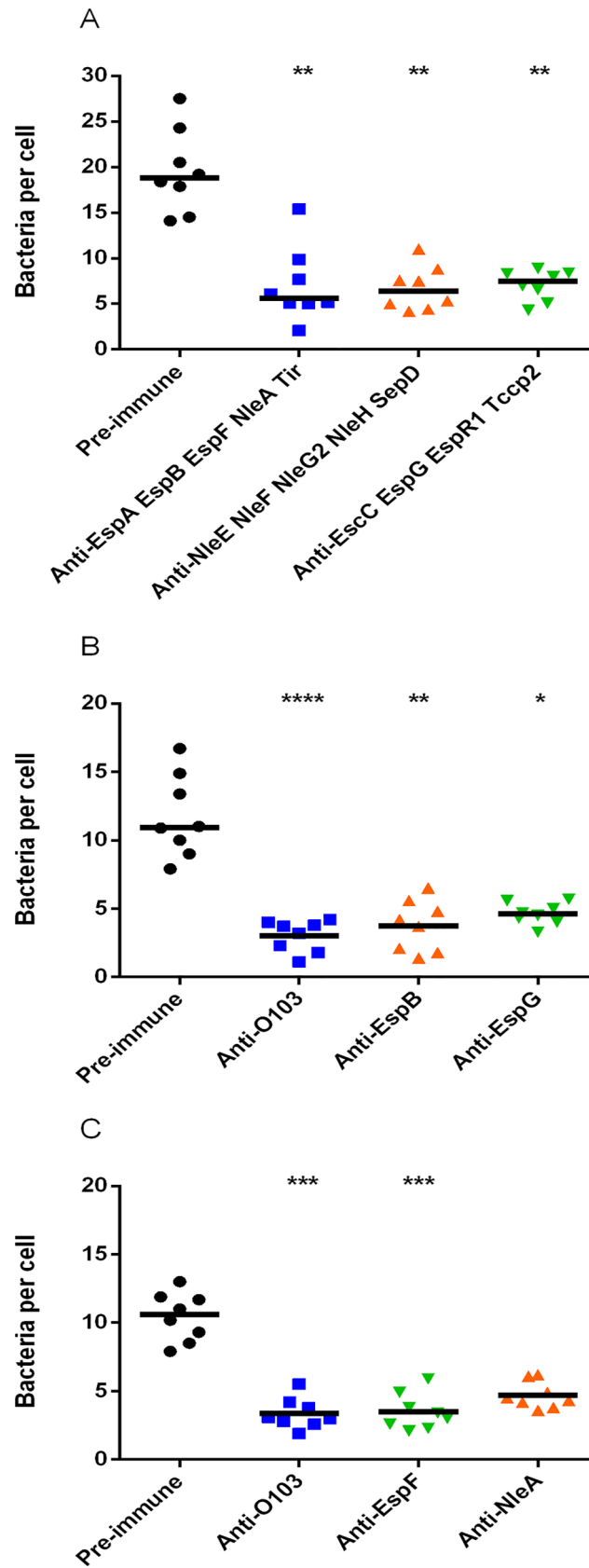
Fig 1. Inhibition of STEC$_{O103}$ adherence to HEp-2 cells by either (A) pooled sera against recombinant STEC$_{O103}$ T3SPs or (B and C) individual serum samples specific for STEC$_{O103}$ recombinant proteins EspB, EspF, EspG or NleA. Anti-O103 refers to antibodies against a secreted fraction of T3SPs from STEC$_{O103}$. Values are expressed as median bacteria per cell from 8 replicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

doi:10.1371/journal.pone.0139803.g001

STEC$_{O103}$ EspA, EspB, EspF, NleA and Tir. Alkaline phosphatase labeled goat anti-mouse or goat anti-rabbit IgG (KPL) antibodies were used as secondary antibodies (1:2000). The membranes were developed using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) salt according to the manufacturer's instructions (Sigma).

Statistical analyses

Statistical Analyses were performed using GraphPad Prism 6.02. Adherence inhibition assays were analyzed using a non-parametric analysis (Kruskal-Wallis test) and individual groups were tested using Dunn's multiple comparison test. Mouse antibody titers were presented as medians plus/minus the 25th and 75th percentile ranges. Differences in immune responses between the vaccine and control groups were tested using non-parametric repeated measures ANOVA. A P value of < 0.05 was considered significant.

Results

Immune responses against STEC$_{O103}$ recombinant proteins in rabbits

Polyclonal sera were raised against 14 STEC$_{O103}$ recombinant proteins in rabbits in order to test the adherence inhibition effect of the sera in vitro and for cross reactivity studies. All the
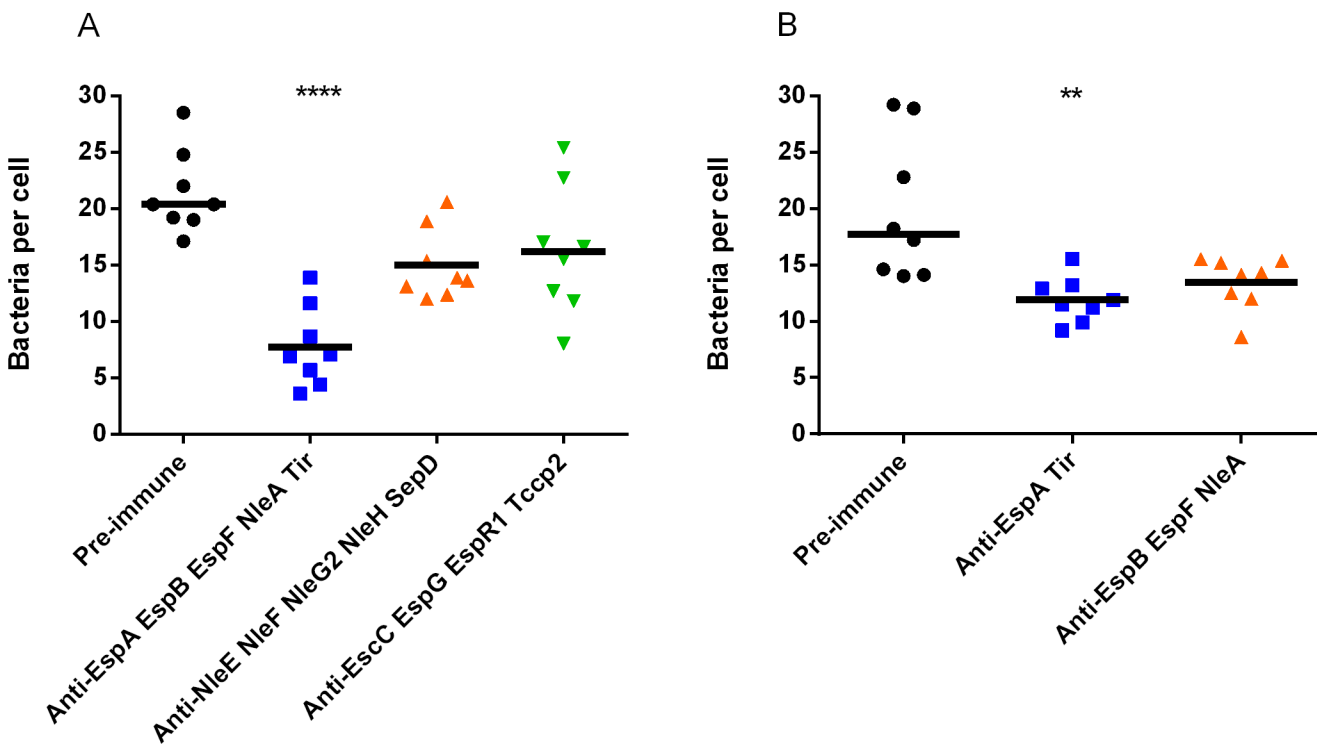

Fig 2. Inhibition of STEC$_{O157}$ adherence to HEp-2 cells by pooled sera against recombinant STEC$_{O103}$ T3SPs. (A) STEC$_{O157}$ adherence to HEp-2 cells was significantly lower in the presence of antibodies against STEC$_{O103}$ EspA, EspB, EspF, NleA and Tir. (B) STEC$_{O157}$ adherence was partially lower in the presence of STEC$_{O103}$ anti-EspA and anti-Tir or anti-EspB, anti-EspF and anti-NleA. Values are expressed as median bacteria per cell from 8 replicates. **, $P < 0.01$; ****, $P < 0.0001$.

doi:10.1371/journal.pone.0139803.g002

recombinant proteins induced a significant IgG specific antibody response as determined by ELISA ([Table 1](#)). The mean IgG titer across all the proteins was 911,801, while NleE had the lowest antibody titer (151,399) and EspR1 had the highest antibody titer (2,771,000).

Antibodies against STEC$_{O103}$ T3SP's inhibit adherence of STEC$_{O103}$

To test the effect of rabbit polyclonal sera against recombinant STEC$_{O103}$ T3SPs on adherence, we used a functional assay where we measured the level of STEC$_{O103}$ adherence to HEp-2 cells. Our results demonstrate that pooled sera against STEC$_{O103}$ recombinant proteins significantly reduced adherence of STEC$_{O103}$ to HEp-2 cells relative to the group incubated with pre-immune sera ([Fig 1A](#)). In order to determine which serum samples were involved in this adherence inhibition effect, we tested specific anti-sera to EspA, EspB, EspF, EspG, EscC, EspR1, NleA, NleE, NleF, NleG2, NleH, SepD, Tir and Tccp2 individually in duplicate. We observed that sera against EspB, EspG, EspF and NleA were involved in blocking adherence (data not shown). To confirm this observation, we performed an adherence inhibition assay where sera against EspB, EspG, EspF and NleA were tested individually with 8 replicates. The data clearly suggest that anti-sera to these four proteins were also highly effective in blocking STEC$_{O103}$ adherence to HEp-2 cells compared to the group treated with pre-immune serum ([Fig 1B and 1C](#)).

Anti- STEC$_{O103}$ T3SP sera have cross-protective potential

In order to determine if antibodies against STEC$_{O103}$ recombinant proteins can block adherence of other STEC serotypes, we evaluated the effect of pooled sera on STEC$_{O157}$ adherence to HEp-2 cells. Interestingly, our results indicate that incubation of STEC$_{O157}$ with anti-sera to STEC$_{O103}$ EspA, EspB, EspF, NleA and Tir significantly lowered adherence to HEp-2 cells, while anti-sera to the other proteins did not have a major effect ([Fig 2A](#)). We further investigated this adherence inhibition effect by testing pooled sera against STEC$_{O103}$ EspA and Tir in one group and sera against EspB, EspF and NleA in another group. Adherence of STEC$_{O157}$ to

Table 1. IgG antibody titers specific for STEC$_{O103}$ recombinant proteins in rabbits.

	Pre-immune	Day 56
Anti-EscC	87.5	241547
Anti-EspA	882.5	838090
Anti-EspB	783.5	1256000
Anti-EspF	711	618333
Anti-EspG	773.5	846100
Anti-EspR1	789.5	2771000
Anti-NleA	2778	1332000
Anti-NleE	555.5	151399
Anti-NleF	2748.5	1036000
Anti-NleG2	652	1358000
Anti-NleH	499.5	614552
Anti-SepD	536.5	483176
Anti-Tccp2	252.5	402873
Anti-Tir	122.5	816244

Antibody titers were determined by setting the cut-off value as the average of the blank and two standard deviations. All serum samples were tested in duplicate wells.

doi:10.1371/journal.pone.0139803.t001

Table 2. Median IgG antibody titers specific for STEC$_{O103}$ EspA, EspB, EspF, NleA or Tir in mice vaccinated with either PBS or a pool of STEC$_{O103}$ recombinant proteins.

Antibody	PBS				Vaccine				P
	Day 0		Day 35		Day 0		Day 35		
Anti-EspA	693	(590–853)	1089	(736–1462)	772	(581–860)	6979	(3109–11618)	0.0007
Anti-EspB	235	(157–304)	600	(353–814)	243	(221–477)	294644	(195617–427747)	<0.0001
Anti-EspF	882	(742–1240)	1232	(973–5538)	1239	(753–1930)	3822	(1067–13092)	0.052
Anti-NleA	481	(161–839)	2734	(422–7809)	552	(250–4314)	1019000	(5364–1385000)	0.0004
Anti-Tir	570	(470–1154)	845	(711–3461)	517	(387–652)	280528	(151906–821988)	<0.0001

Values are expressed as median titers. Numbers in parentheses represent the 25th– 75th percentile. Antibody titers were determined by setting the cut-off value as the average of the blank and two standard deviations. All serum samples were tested in duplicate wells.

doi:10.1371/journal.pone.0139803.t002

HEp-2 cells was lower in both groups relative to the control group (Fig 2B) but not to the same level as in the pooled group (Fig 2A). These results suggest that antibodies to STEC$_{O103}$ EspA, EspB, EspF, NleA and Tir proteins have a combined effect on blocking STEC$_{O157}$ adherence and that they have cross-protective potential.

Immunization of mice with T3SP's from STEC$_{O103}$ induces a strong humoral response but does not affect fecal shedding

In order to test the protective capacity of STEC$_{O103}$ effectors, mice were vaccinated subcutaneously with a pool of recombinant proteins and were subsequently infected with STEC$_{O103}$ by oral challenge. Two weeks following the booster immunization, significant EspA-, EspB-, NleA- and Tir–specific IgG titers were detected in the sera relative to the control group (Table 2). In contrast, immunization with EspF elicited a weak IgG specific serum response. To assess the protective capacity of our vaccine, fecal shedding of STEC$_{O103}$ was monitored over 21 days post challenge. The levels of STEC$_{O103}$ were similar in both vaccinates and non-vaccinates throughout the duration of the study, suggesting that antibodies against the antigens used for immunization did not prevent STEC$_{O103}$ from persisting in the intestine (Fig 3), or that the response was not of sufficient magnitude.

STEC$_{O26}$ and STEC$_{O111}$ T3SS proteins display significant cross-reactivity with anti-sera to the corresponding STEC$_{O103}$ proteins

The cross-reactivity of sera against STEC$_{O103}$ T3SS proteins with other STEC serotypes including STEC$_{O26}$ and STEC$_{O111}$, was first tested by western blotting using rabbit polyclonal sera. Our results indicate that EspB$_{O111}$, EspF$_{O111}$ and NleA$_{O111}$ reacted strongly with anti-sera to the corresponding STEC$_{O103}$ proteins, while Tir$_{O111}$ displayed a weaker reaction and EspA$_{O111}$ did not react (Fig 4A). The western blot profile for STEC$_{O26}$ proteins was similar with respect to EspB$_{O26}$ and EspF$_{O26}$. However, EspA$_{O26}$ also reacted strongly, unlike EspA$_{O111}$, while NleA$_{O26}$ did not react (Fig 4B). Subsequently, sera from mice immunized with a pool of STEC$_{O103}$ recombinant proteins was used to study the cross-reactivity with the equivalent STEC$_{O26}$ and STEC$_{O111}$ proteins. The results indicate that EspB$_{O26}$ reacted strongly with the anti-sera while EspA$_{O26}$, EspF$_{O26}$ and NleA$_{O26}$ did not (Fig 5A). In contrast, the western blot profile for the STEC$_{O111}$ recombinant proteins showed a significant degree of cross reactivity for EspB$_{O111}$, NleA$_{O111}$ and Tir$_{O111}$ (Fig 5B). Taken together, the results suggest that EspB$_{O103}$, NleA$_{O103}$, and Tir$_{O103}$ possess significant cross-reactive properties with the corresponding
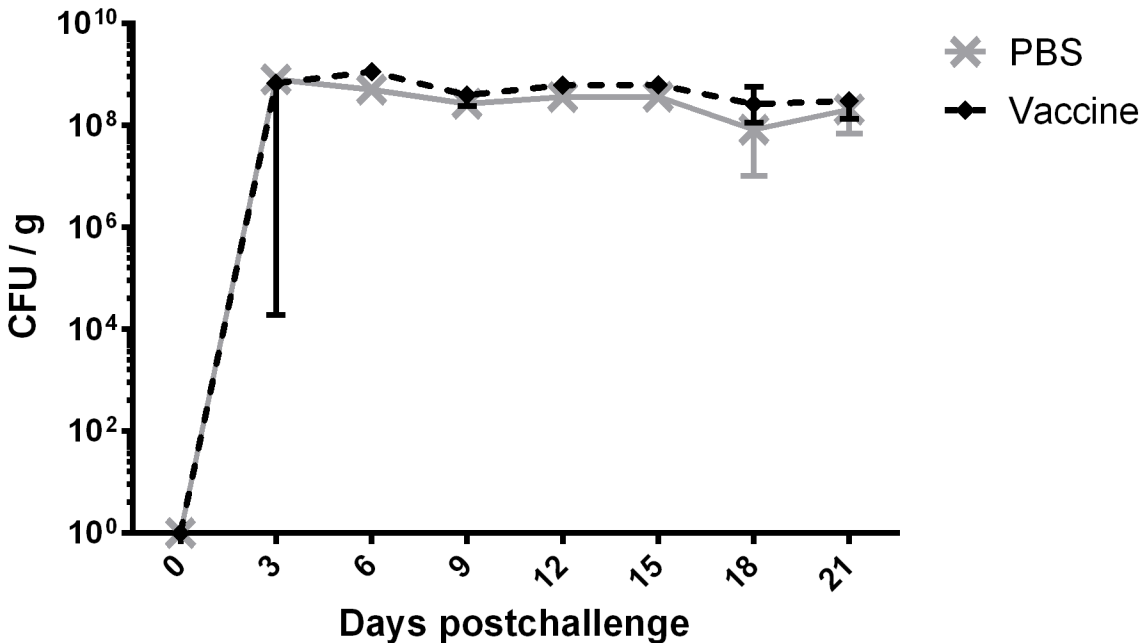
Fig 3. STEC$_{O103}$ shedding in feces following oral challenge in mice. Mice were immunized subcutaneously with either PBS (control) or a pool of STEC$_{O103}$ EspA, EspB, EspF, NleA and Tir followed by a booster immunization three weeks later. Two weeks after the second immunization, mice were orally challenged with 10^9 cfu of STEC$_{O103}$. N = 12 for both groups. Values are expressed as median cfu per gram of feces.

proteins from STEC$_{O26}$ and STEC$_{O111}$. Hence, these proteins may form the basis of a cross-protective vaccine that confers protection against multiple STEC serotypes.

Discussion

Many efforts have been made to develop STEC$_{O157}$ vaccines using the T3SS proteins as targets in order to reduce the levels of the pathogen in cattle [5,12]. However, these vaccination strategies provide limited protection as they are directed only against STEC$_{O157}$ and they are limited in their benefit [20,21]. Non-O157 STEC serotypes are more prevalent in other parts of the world [1] and with the rise in non-O157 STEC infections in humans [26] as well as the increase in the prevalence of these serotypes in cattle [27], a vaccine that can confer protection against multiple serotypes is more desirable. The aim of this study was to determine if STEC$_{O103}$ T3SS proteins could provide protection against STEC$_{O103}$ as well as other heterologous serotypes using adherence-inhibition assays and the streptomycin-treated mouse model.

We used STEC$_{O103}$ T3SS proteins as targets for a potentially cross protective vaccine since the T3SS proteins encoded by this serotype have previously been shown to have the highest degree of cross reactivity with STEC$_{O157}$, relative to STEC$_{O26}$ and STEC$_{O111}$ [20,21]. Based on this, we over-expressed and purified STEC$_{O103}$ EscC, EspA, EspB, EspF, EspG, EspR1, NleA, NleE, NleF, NleG2, NleH, SepD, Tccp2 and Tir recombinant proteins. In order to test the protective capacity of these proteins, we first examined the effect of rabbit polyclonal sera against the candidate proteins in vitro using a HEp-2 cell adherence inhibition assay which has been successfully used as a functional assay to study STEC adherence [21,28]. This, in turn, may reflect the effect of antibodies on intestinal colonization. Our results demonstrate that pooled sera against STEC$_{O103}$ recombinant proteins significantly inhibited STEC$_{O103}$ adherence. This is in agreement with the findings of Asper *et al*, where anti-sera to all STEC$_{O103}$ secreted proteins blocked adherence by this serotype [21]. We also show for the first time that sera against
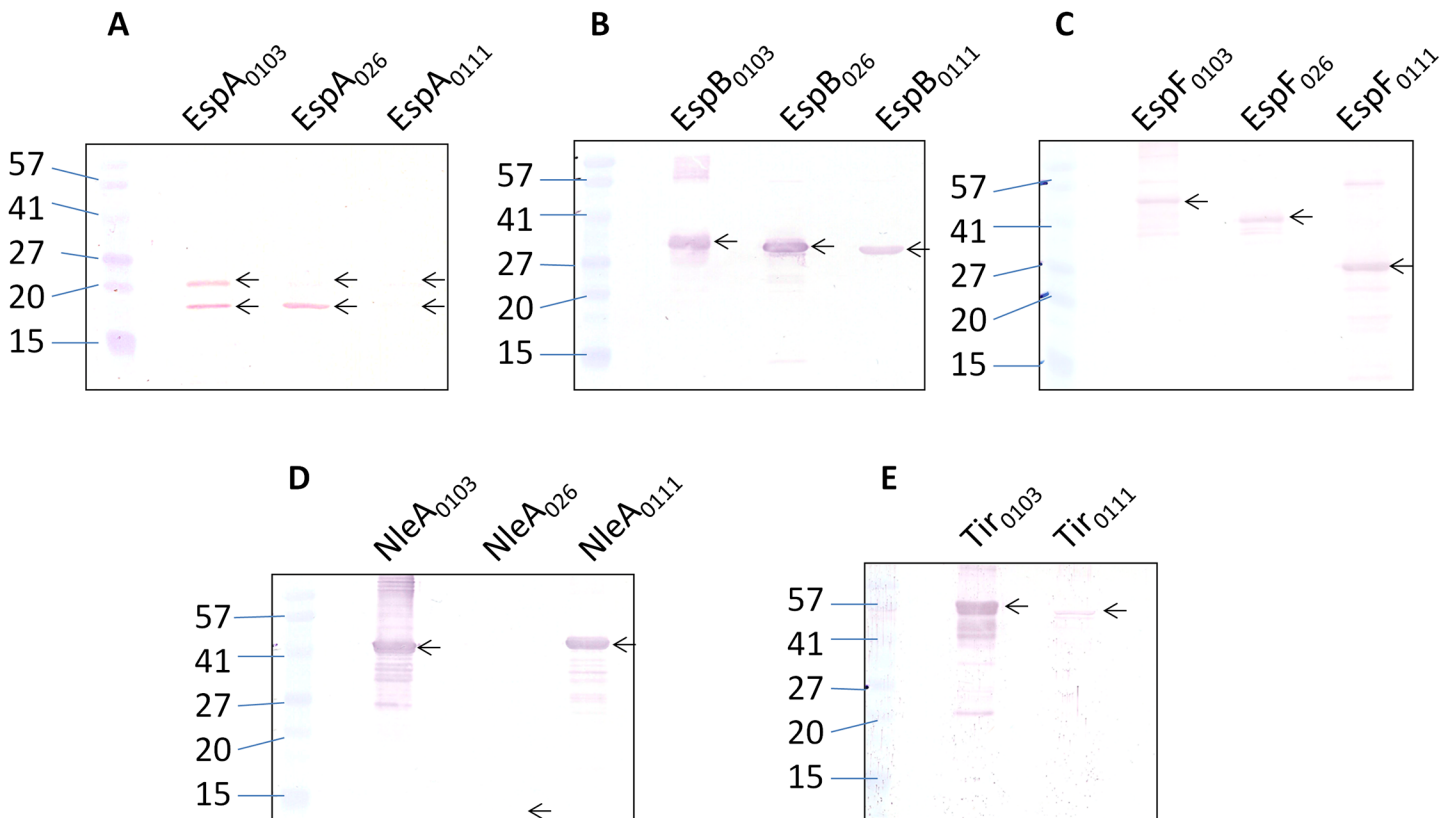
Fig 4. Cross-reactivity of $STEC_{O103}$ EspA, EspB, EspF, NleA and Tir specific rabbit polyclonal sera with the corresponding $STEC_{O26}$ and $STEC_{O111}$ recombinant proteins. (A) Western blot using anti-EspA. Lane 1, marker; Lane 2, $EspA_{O103}$ (20.5 kDa); Lane 3, $EspA_{O26}$ (20.5 kDa); Lane 4, $EspA_{O111}$(20.5 kDa). (B) Western blot using anti-EspB. Lane 1, marker; Lane 2, $EspB_{O103}$ (33.1 kDa); Lane 3, $EspB_{O26}$ (33.2 kDa); Lane 4, $EspB_{O111}$ (32.8 kDa). (C) Western blot using anti-EspF. Lane 1, marker; Lane 2, $EspF_{O103}$ (57 kDa); Lane 3, $EspF_{O26}$ (40 kDa); Lane 4, $EspF_{O111}$ (27 kDa). (D) Western blot using anti-NleA. Lane 1, marker; Lane 2, $NleA_{O103}$ (44 kDa); Lane 3, $NleA_{O26}$ (11.7 kDa); Lane 4, $NleA_{O111}$ (47.5 kDa). (E) Western blot using anti- Tir. Lane 1, marker; Lane 2, Tir_{O103} (56 kDa); Lane 3, Tir_{O111} (56.9 kDa).

doi:10.1371/journal.pone.0139803.g004

individual $STEC_{O103}$ recombinant proteins including, EspB, EspF, EspG and NleA, inhibited adherence of the bacteria to HEp-2 cells. Interestingly, pooled anti-sera to $STEC_{O103}$ recombinant proteins EspA, EspB, EspF, NleA and Tir were able to block adherence of $STEC_{O157}$, suggesting that these candidate proteins may provide protection against multiple STEC serotypes. However, it appears that the inhibition of $STEC_{O157}$, unlike that of $STEC_{O103}$, was due to a combination of $STEC_{O103}$ anti-sera since there was reduced inhibition of $STEC_{O157}$ once the pooled anti-sera were divided into two groups. Taken together, this is the first report which illustrates that sera against $STEC_{O103}$ T3SS recombinant proteins can block adherence of $STEC_{O157}$ to HEp-2 cells.

The streptomycin-treated mouse model [24,29] was used to test the efficacy of the identified candidate $STEC_{O103}$ recombinant proteins as antigens for protection against $STEC_{O103}$. This model was chosen since it has been widely used by various groups to test their STEC vaccines prior to conducting studies in cattle [25,30,31,32,33]. The mice developed strong serum IgG specific titers against $EspA_{O103}$, $EspB_{O103}$, $NleA_{O103}$ and Tir_{O103} following immunization, while the response to the corresponding EspF recombinant protein was weak. The weak response to $EspF_{O103}$ is in line with what was observed for $EspF_{O157}$ in a previous study published by our group [20]. Immunization with $STEC_{O103}$ recombinant proteins did not affect $STEC_{O103}$ fecal shedding over the duration of the experiment relative to the control group.
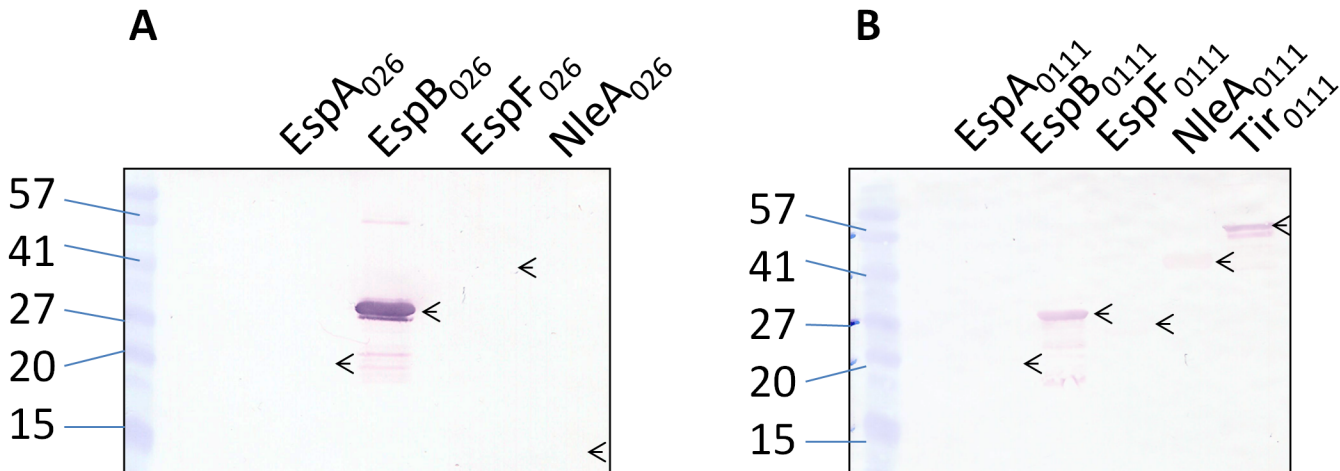
Fig 5. Cross-reactivity of STEC$_{O103}$ EspA, EspB, EspF, NleA and Tir specific mouse polyclonal sera with the corresponding STEC$_{O26}$ and STEC$_{O111}$ recombinant proteins. Western blot using sera from mice immunized with a combination of STEC$_{O103}$ EspA, EspB, EspF, NleA and Tir to test the cross-reactivity with: (A) STEC$_{O26}$ proteins. Lane 1, marker; Lane 2, EspA$_{O26}$ (20.5 kDa); Lane 3, EspB$_{O26}$ (33.2 kDa); Lane 4, EspF$_{O26}$ (40 kDa); and Lane 5, NleA$_{O26}$ (11.7 kDa). (B) STEC$_{O111}$ proteins. Lane 1, marker; Lane 2, EspA$_{O111}$ (20.5 kDa); Lane 3, EspB$_{O111}$ (32.8 kDa); Lane 4, EspF $_{O111}$ (27 kDa); Lane 5, NleA $_{O111}$ (47.5 kDa); and Lane 6, Tir$_{O111}$ (56.9 kDa).

doi:10.1371/journal.pone.0139803.g005

This was unexpected since similar STEC$_{O157}$ based vaccines have been highly effective in mice [30,33]. In addition, a recent vaccination study by our group illustrated that a combination of nine STEC$_{O157}$ recombinant proteins was highly effective in controlling STEC$_{O157}$ fecal shedding in mice (data not shown). It is possible that our STEC$_{O103}$ vaccine may have been more effective against intestinal colonization had it been administered intranasaly. However, both subcutaneous and intranasal immunization of mice with an extract of STEC$_{O157}$ secreted proteins as well as individual recombinant proteins have proven to be highly effective in controlling STEC$_{O157}$ shedding [30]. In addition, the lack of a robust immune response against EspA may have contributed to the persistence of STEC$_{O103}$ in the intestines. Alternatively, since very little work has been done on STEC$_{O103}$ in mice, we speculate that the T3SS may play a different role in STEC$_{O103}$ infection in mice. Therefore, further analysis of the STEC$_{O103}$ T3SS may be required in mice, while a similar vaccine study should be performed in cattle with STEC$_{O103}$.

The serological cross reactivity of STEC$_{O103}$ recombinant proteins EspA, EspB, EspF, NleA and Tir with the corresponding STEC$_{O26}$ and STEC$_{O111}$ proteins was analyzed by western blotting. Overall our results indicate that there was significant cross reactivity of the STEC$_{O26}$ recombinant proteins, EspA$_{O26}$, EspB$_{O26}$ and EspF$_{O26}$ when rabbit polyclonal sera were used. These observations are supported by the protein sequence homology of the STEC$_{O26}$ proteins to STEC$_{O103}$: EspA$_{O26}$ (92%), EspB$_{O26}$ (99%) and EspF$_{O26}$ (91%). The fact that NleA$_{O26}$ did not cross react was not unexpected since the STEC$_{O26}$ genome contains an NleA-like gene which encodes for an 11 kDa protein, while the actual size of NleA$_{O103}$ is 44 kDa. Therefore, sequence homology between NleA$_{O103}$ and NleA$_{O26}$ is expected to be low (58%) with few shared epitopes, if any. We did not show the results for Tir$_{O26}$ since we were unable to express or purify this protein despite numerous attempts. This may be explained by the fact that Tir$_{O26}$ may require co-expression and co-purification with a chaperone [34]. The western blot profile for STEC$_{O111}$ recombinant proteins EspB$_{O111}$, EspF$_{O111}$, NleA$_{O111}$ showed a high degree of cross reactivity with the corresponding sera, while there was lower cross reactivity with Tir$_{O111}$. This is consistent with the observed sequence homologies between the STEC$_{O103}$ and STEC$_{O111}$ proteins: EspB$_{O111}$ (71%), EspF$_{O111}$ (70%), NleA (83%) and Tir (65%). The fact that EspA$_{O111}$ did not react to sera against EspA$_{O103}$ was surprising since EspA$_{O111}$ shares greater

than 81% sequence homology to EspA$_{O103}$. The serological cross reactivity of the STEC serotypes O26 and O111 recombinant proteins was remarkably lower when mouse polyclonal sera were used. The difference in the results may be due to differences in recognition of epitopes by the mouse and rabbit immune systems. Overall, the data from both cross reactivity studies suggests that EspB$_{O103}$, NleA$_{O103}$ and Tir$_{O103}$ are highly cross reactive and have the potential to form an efficacious recombinant vaccine that protects cattle not only against STEC$_{O103}$ but other STEC serotypes as well. This finding is supported by two recent studies which demonstrate that EspB$_{O157}$ and Tir$_{O157}$ are immunogenic and protective in cattle against STEC$_{O157}$ [35,36]. Although these studies provide important information about STEC$_{O157}$, this can be used as a basis for conducting similar studies with STEC$_{O103}$ to test for cross serotype protection.

Vaccination with a commercially available STEC$_{O157}$ T3SS vaccine (Econiche™) is an effective strategy to control STEC shedding in cattle [37]. Many recent studies have proven that this preslaughter intervention does lead to reduced levels of this pathogen in cattle [38,39]. Moreover, Mathews *et al* have recently predicted that vaccination of cattle against STEC$_{O157}$ will have a significant impact on public health by lowering human STEC infections by 85% [40]. Our in vitro results are the first steps towards a vaccine that may provide protection against multiple STEC serotypes, which is highly desirable for both North America as well as other regions where non-O157 STEC serotypes are more prevalent. The STEC$_{O157}$ SRP$^{®}$ vaccine (contains siderophore and porin proteins) has also shown to be effective in reducing fecal shedding in cattle [41,42]. However, this vaccine also confers limited serotype protection like the Econiche™ vaccine [43,44]. Taken together, the need for an STEC vaccine that provides protection against more than one serotype is required and our in vitro results suggest that STEC$_{O103}$ may be a likely candidate, though further testing is required in cattle.

Acknowledgments

Author Contributions

References

1. Ho NK, Henry AC, Johnson-Henry K, Sherman PM (2013) Pathogenicity, host responses and implications for management of enterohemorrhagic Escherichia coli O157:H7 infection. Can J Gastroenterol 27: 281–285. PMID: 23712303

2. Beutin L, Kaulfuss S, Herold S, Oswald E, Schmidt H (2005) Genetic analysis of enteropathogenic and enterohemorrhagic Escherichia coli serogroup O103 strains by molecular typing of virulence and housekeeping genes and pulsed-field gel electrophoresis. J Clin Microbiol 43: 1552–1563. PMID: 15814965

3. Schimmer B, Nygard K, Eriksen HM, Lassen J, Lindstedt BA, Brandal LT, et al. (2008) Outbreak of haemolytic uraemic syndrome in Norway caused by stx2-positive Escherichia coli O103:H25 traced to cured mutton sausages. BMC Infect Dis 8: 41. doi: 10.1186/1471-2334-8-41 PMID: 18387178

4. Gyles CL (2007) Shiga toxin-producing Escherichia coli: an overview. J Anim Sci 85: E45—62. PMID: 17085726

5. Vande Walle K, Vanrompay D, Cox E (2013) Bovine innate and adaptive immune responses against Escherichia coli O157:H7 and vaccination strategies to reduce faecal shedding in ruminants. Vet Immunol Immunopathol 152: 109–120. doi: 10.1016/j.vetimm.2012.09.028 PMID: 23084625

6. Garmendia J, Frankel G, Crepin VF (2005) Enteropathogenic and enterohemorrhagic Escherichia coli infections: translocation, translocation, translocation. Infect Immun 73: 2573–2585. PMID: 15845459

7. Tobe T, Beatson SA, Taniguchi H, Abe H, Bailey CM, Fivian A, et al. (2006) An extensive repertoire of type III secretion effectors in Escherichia coli O157 and the role of lambdoid phages in their dissemination. Proc Natl Acad Sci U S A 103: 14941–14946. PMID: 16990433

8. Dziva F, van Diemen PM, Stevens MP, Smith AJ, Wallis TS (2004) Identification of Escherichia coli O157: H7 genes influencing colonization of the bovine gastrointestinal tract using signature-tagged mutagenesis. Microbiology 150: 3631–3645. PMID: 15528651

9. Naylor SW, Roe AJ, Nart P, Spears K, Smith DG, Low JC, et al. (2005) Escherichia coli O157: H7 forms attaching and effacing lesions at the terminal rectum of cattle and colonization requires the LEE4 operon. Microbiology 151: 2773–2781. PMID: 16079353

10. Sharma VK, Sacco RE, Kunkle RA, Bearson SM, Palmquist DE (2012) Correlating levels of type III secretion and secreted proteins with fecal shedding of Escherichia coli O157:H7 in cattle. Infect Immun 80: 1333–1342. doi: 10.1128/IAI.05869-11 PMID: 22252878

11. Peterson RE, Klopfenstein TJ, Moxley RA, Erickson GE, Hinkley S, Bretschneider G, et al. (2007) Effect of a vaccine product containing type III secreted proteins on the probability of Escherichia coli O157:H7 fecal shedding and mucosal colonization in feedlot cattle. J Food Prot 70: 2568–2577. PMID: 18044436

12. Potter AA, Klashinsky S, Li Y, Frey E, Townsend H, Rogan D, et al. (2004) Decreased shedding of Escherichia coli O157:H7 by cattle following vaccination with type III secreted proteins. Vaccine 22: 362–369. PMID: 14670317

13. Van Donkersgoed J, Hancock D, Rogan D, Potter AA (2005) Escherichia coli O157:H7 vaccine field trial in 9 feedlots in Alberta and Saskatchewan. Can Vet J 46: 724–728. PMID: 16187717

14. Peterson RE, Klopfenstein TJ, Moxley RA, Erickson GE, Hinkley S, Rogan D, et al. (2007) Efficacy of dose regimen and observation of herd immunity from a vaccine against Escherichia coli O157:H7 for feedlot cattle. J Food Prot 70: 2561–2567. PMID: 18044435

15. Smith DR, Moxley RA, Peterson RE, Klopfenstein TJ, Erickson GE, Clowser SL (2008) A two-dose regimen of a vaccine against Escherichia coli O157:H7 type III secreted proteins reduced environmental transmission of the agent in a large-scale commercial beef feedlot clinical trial. Foodborne Pathog Dis 5: 589–598. doi: 10.1089/fpd.2008.0080 PMID: 18681791

16. Smith DR, Moxley RA, Peterson RE, Klopfenstein TJ, Erickson GE, Bretschneider G, et al. (2009) A two-dose regimen of a vaccine against type III secreted proteins reduced Escherichia coli O157:H7 colonization of the terminal rectum in beef cattle in commercial feedlots. Foodborne Pathog Dis 6: 155–161. doi: 10.1089/fpd.2008.0136 PMID: 19105625

17. Smith DR, Moxley RA, Klopfenstein TJ, Erickson GE (2009) A randomized longitudinal trial to test the effect of regional vaccination within a cattle feedyard on Escherichia coli O157:H7 rectal colonization, fecal shedding, and hide contamination. Foodborne Pathog Dis 6: 885–892. doi: 10.1089/fpd.2009.0299 PMID: 19618995

18. Moxley RA, Smith DR, Luebbe M, Erickson GE, Klopfenstein TJ, Rogan D (2009) Escherichia coli O157:H7 vaccine dose-effect in feedlot cattle. Foodborne Pathog Dis 6: 879–884. doi: 10.1089/fpd.2009.0297 PMID: 19737064

19. Allen KJ, Rogan D, Finlay BB, Potter AA, Asper DJ (2011) Vaccination with type III secreted proteins leads to decreased shedding in calves after experimental infection with Escherichia coli O157. Can J Vet Res 75: 98–105. PMID: 21731179

20. Asper DJ, Karmali MA, Townsend H, Rogan D, Potter AA (2011) Serological response of Shiga toxin-producing Escherichia coli type III secreted proteins in sera from vaccinated rabbits, naturally infected cattle, and humans. Clin Vaccine Immunol 18: 1052–1057. doi: 10.1128/CVI.00068-11 PMID: 21593239

21. Asper DJ, Sekirov I, Finlay BB, Rogan D, Potter AA (2007) Cross reactivity of enterohemorrhagic Escherichia coli O157:H7-specific sera with non-O157 serotypes. Vaccine 25: 8262–8269. PMID: 17980466

22. Karmali MA, Mascarenhas M, Shen S, Ziebell K, Johnson S, Reid-Smith R, et al. (2003) Association of genomic O island 122 of Escherichia coli EDL 933 with verocytotoxin-producing Escherichia coli seropathotypes that are linked to epidemic and/or serious disease. J Clin Microbiol 41: 4930–4940. PMID: 14605120

23. Tarr PI, Neill MA, Clausen CR, Newland JW, Neill RJ, Moseley SL (1989) Genotypic variation in pathogenic Escherichia coli O157:H7 isolated from patients in Washington, 1984–1987. J Infect Dis 159: 344–347. PMID: 2644374

24. Wadolkowski EA, Burris JA, O'Brien AD (1990) Mouse model for colonization and disease caused by enterohemorrhagic Escherichia coli O157:H7. Infect Immun 58: 2438–2445. PMID: 2196227

25. Zhang XH, He KW, Zhang SX, Lu WC, Zhao PD, Luan XT, et al. (2011) Subcutaneous and intranasal immunization with Stx2B-Tir-Stx1B-Zot reduces colonization and shedding of Escherichia coli O157: H7 in mice. Vaccine 29: 3923–3929. doi: 10.1016/j.vaccine.2011.02.007 PMID: 21338683

26. Smith JL, Fratamico PM, Gunther NWt (2014) Shiga toxin-producing Escherichia coli. Adv Appl Microbiol 86: 145–197. doi: 10.1016/B978-0-12-800262-9.00003-2 PMID: 24377855

27. Gill A, Gill CO (2010) Non-O157 verotoxigenic Escherichia coli and beef: a Canadian perspective. Can J Vet Res 74: 161–169. PMID: 20885839

28. La Ragione RM, Patel S, Maddison B, Woodward MJ, Best A, Whitelam GC, et al. (2006) Recombinant anti-EspA antibodies block Escherichia coli O157:H7-induced attaching and effacing lesions in vitro. Microbes Infect 8: 426–433. PMID: 16298154

29. Mohawk KL, O'Brien AD (2011) Mouse models of Escherichia coli O157:H7 infection and shiga toxin injection. J Biomed Biotechnol 2011: 258185. doi: 10.1155/2011/258185 PMID: 21274267

30. Babiuk S, Asper DJ, Rogan D, Mutwiri GK, Potter AA (2008) Subcutaneous and intranasal immunization with type III secreted proteins can prevent colonization and shedding of Escherichia coli O157:H7 in mice. Microb Pathog 45: 7–11. doi: 10.1016/j.micpath.2008.01.005 PMID: 18487034

31. Gao X, Cai K, Li T, Wang Q, Hou X, Tian R, et al. (2011) Novel fusion protein protects against adherence and toxicity of enterohemorrhagic Escherichia coli O157:H7 in mice. Vaccine 29: 6656–6663. doi: 10.1016/j.vaccine.2011.06.106 PMID: 21742003

32. Cai K, Gao X, Li T, Wang Q, Hou X, Tu W, et al. (2011) Enhanced immunogenicity of a novel Stx2Am-Stx1B fusion protein in a mice model of enterohemorrhagic Escherichia coli O157:H7 infection. Vaccine 29: 946–952. doi: 10.1016/j.vaccine.2010.11.035 PMID: 21134452

33. Amani J, Salmanian AH, Rafati S, Mousavi SL (2010) Immunogenic properties of chimeric protein from espA, eae and tir genes of Escherichia coli O157:H7. Vaccine 28: 6923–6929. doi: 10.1016/j.vaccine. 2010.07.061 PMID: 20709010

34. Abe A, de Grado M, Pfuetzner RA, Sanchez-Sanmartin C, Devinney R, Puente JL, et al. (1999) Enteropathogenic Escherichia coli translocated intimin receptor, Tir, requires a specific chaperone for stable secretion. Mol Microbiol 33: 1162–1175. PMID: 10510231

35. McNeilly TN, Mitchell MC, Rosser T, McAteer S, Low JC, Smith DG, et al. (2010) Immunization of cattle with a combination of purified intimin-531, EspA and Tir significantly reduces shedding of Escherichia coli O157:H7 following oral challenge. Vaccine 28: 1422–1428. doi: 10.1016/j.vaccine.2009.10.076 PMID: 19903545

36. Vilte DA, Larzabal M, Garbaccio S, Gammella M, Rabinovitz BC, Elizondo AM, et al. (2011) Reduced faecal shedding of Escherichia coli O157:H7 in cattle following systemic vaccination with gamma-intimin C(2)(8)(0) and EspB proteins. Vaccine 29: 3962–3968. doi: 10.1016/j.vaccine.2011.03.079 PMID: 21477674

37. Vogstad AR, Moxley RA, Erickson GE, Klopfenstein TJ, Smith DR (2014) Stochastic simulation model comparing distributions of STEC O157 faecal shedding prevalence between cattle vaccinated with type III secreted protein vaccines and non-vaccinated cattle. Zoonoses Public Health 61: 283–289. doi: 10. 1111/zph.12069 PMID: 23826923

38. Vogstad AR, Moxley RA, Erickson GE, Klopfenstein TJ, Smith DR (2013) Assessment of heterogeneity of efficacy of a three-dose regimen of a type III secreted protein vaccine for reducing STEC O157 in feces of feedlot cattle. Foodborne Pathog Dis 10: 678–683. doi: 10.1089/fpd.2012.1374 PMID: 23692077

39. Boland KG, Hayles AN, Miller CB, Kerr T, Brown WC, Lahmers KK (2013) Regional immune response to immunization with Escherichia coli O157:H7-derived intimin in cattle. Clin Vaccine Immunol 20: 562–571. doi: 10.1128/CVI.00743-12 PMID: 23408521

40. Matthews L, Reeve R, Gally DL, Low JC, Woolhouse ME, McAteer SP, et al. (2013) Predicting the public health benefit of vaccinating cattle against Escherichia coli O157. Proc Natl Acad Sci U S A 110: 16265–16270. doi: 10.1073/pnas.1304978110 PMID: 24043803

41. Fox JT, Thomson DU, Drouillard JS, Thornton AB, Burkhardt DT, Emery DA, et al. (2009) Efficacy of Escherichia coli O157:H7 siderophore receptor/porin proteins-based vaccine in feedlot cattle naturally shedding E. coli O157. Foodborne Pathog Dis 6: 893–899. doi: 10.1089/fpd.2009.0336 PMID: 19737065

42. Thomson DU, Loneragan GH, Thornton AB, Lechtenberg KF, Emery DA, Burkhardt DT, et al. (2009) Use of a siderophore receptor and porin proteins-based vaccine to control the burden of Escherichia coli O157:H7 in feedlot cattle. Foodborne Pathog Dis 6: 871–877. doi: 10.1089/fpd.2009.0290 PMID: 19737063

43. Cernicchiaro N, Renter DG, Cull CA, Paddock ZD, Shi X, Nagaraja TG (2014) Fecal shedding of non-O157 serogroups of Shiga toxin-producing Escherichia coli in feedlot cattle vaccinated with an Escherichia coli O157:H7 SRP vaccine or fed a Lactobacillus-based direct-fed microbial. J Food Prot 77: 732–737. doi: 10.4315/0362-028X.JFP-13-358 PMID: 24780326

44. Paddock ZD, Renter DG, Cull CA, Shi X, Bai J, Nagaraja TG (2014) Escherichia coli O26 in feedlot cattle: fecal prevalence, isolation, characterization, and effects of an E. coli O157 vaccine and a direct-fed microbial. Foodborne Pathog Dis 11: 186–193. doi: 10.1089/fpd.2013.1659 PMID: 24286301