Serological Response of Shiga Toxin-Producing Escherichia coli Type III Secreted Proteins in Sera from Vaccinated Rabbits, Naturally Infected Cattle, and Humans

David J. Asper, Mohamed A. Karmali, Hugh Townsend, Dragan Rogan, and Andrew A. Potter

Vaccine & Infectious Disease Organization, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5E3; Laboratory for Foodborne Zoonoses, Guelph, Ontario, Canada N1G 3W4; and Bioniche Life Sciences, Belleville, Ontario, Canada K8N 1E2

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Escherichia coli O157:H7 is an important zoonotic pathogen, causing hemolytic uremic syndrome (HUS). The colonization of cattle and human hosts is mediated through the action of effectors secreted via a type III secretion system (T3SS). The structural genes for the T3SS and many of the secreted effectors are located on a pathogenicity island called the locus of enterocyte effacement (LEE). We cloned and expressed the genes coding for 66 effectors and purified each to measure the cross-reactivity of type III secreted proteins from Shiga toxin-producing Escherichia coli (STEC) serotypes. These included 37 LEE-encoded proteins and 29 non-LEE effectors. The serological response against each protein was measured by Western blot analysis and enzyme-linked immunosorbent assay (ELISA) using sera from rabbits immunized with type III secreted proteins (T3SPs) from four STEC serotypes, experimentally infected cattle, and human sera from six HUS patients. Twenty proteins were recognized by at least one of the STEC T3SP-vaccinated rabbits by Western blotting. Several structural proteins (EspA, EspB, and EspD) and a number of effectors (Tir, NleA, and TccP) were recognized by O26-, O103-, O111-, and O157-specific sera. Sera from experimentally infected cattle and HUS patients were tested using an ELISA against each of the proteins. Tir, EspB, EspD, EspA, and NleA were recognized by the majority of the samples tested. A number of other proteins also were recognized by individual serum samples. Overall, proteins such as Tir, EspB, EspD, NleA, and EspA were highly immunogenic in vaccinated and naturally infected subjects and could be candidates for a cross-protective STEC vaccine.

Shiga toxin-producing Escherichia coli (STEC) strains are an important group of zoonotic pathogens that are responsible for hemorrhagic colitis and hemolytic uremic syndrome (HUS) (12, 13, 23). Hemolytic uremic syndrome is attributed to the action of Shiga toxins (Stx1 and Stx2), identified first in Shigella dysenteriae, produced by STEC either alone or in combination (15, 25). Hemolytic uremic syndrome is also the leading cause of acute renal failure in children worldwide.

The most common STEC serotype in North America is O157, where an estimated 73,000 illnesses occur each year in the United States, resulting in 2,000 hospitalizations and 60 deaths (21). The most frequent non-O157 serotypes responsible for disease are O26, O103, O111, and O145, with numerous outbreaks reported worldwide. In Denmark it is estimated that 68% of STEC infections resulted from non-O157 serotypes (24). It also is estimated that 58% of all cases in Argentina, which has the highest reported frequency of HUS worldwide, result from infection by non-O157 serotypes (19, 28).

Cattle are the main reservoir for STEC, and the organism does not cause clinical disease in them. The colonization of both cattle and human hosts is mediated through the action of effector molecules secreted through a type III secretion system (T3SS) (7, 8). These effectors contribute to the formation of attaching and effacing (A/E) lesions, which are the hallmark of STEC infection (9). The genes which express the structural proteins of the T3SS and many of its effectors are located on a pathogenicity island called the locus of enterocyte effacement (LEE) (4). Many of these proteins, such as Tir, EspA, EspB, and EspD, are critical for the virulence of STEC (7, 22). However, the discovery of non-LEE effectors, such as NleA, TccP, and NleB, whose genes are located in small pathogenicity islands, and prophages, also has been shown to play an important role in the colonization and virulence of STEC (6, 14, 22, 27, 31).

A number of experimental vaccines based on LEE proteins have been tested. The vaccination of pregnant dams using intimin from STEC O157 protected suckling piglets against challenge (2), yet a cross-protective vaccine based on this protein would be challenging, as more than 17 serologically distinct variants have been identified (5). Potter and colleagues demonstrated that vaccination with secreted proteins from STEC O157:H7 was able to significantly reduce the number of bacteria shed, as well as the number of shedding animals in an experimental setting (26). However, vaccination using T3SPs appears to be serotype specific (1, 20). At present, the repertoire of T3SPs in the supernatant which are harvested for vaccination, as well as their immunogenic properties, are unknown. In a natural infection, it is unclear which T3SPs are secreted by STEC and recognized by cattle and human hosts.

In this study, we have cloned and expressed the genes coding for 66 structural and effector proteins, which include 37 LEE-encoded proteins and 29 non-LEE effectors to assess their immunological cross-reactivity using sera from vaccinated and naturally infected animals.
naturally infected animals as well as human sera from HUS patients.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study included E. coli EDL933 (O157:H7) (29), CL101 (O111:NM), CL9 (O26:H11), and N01-2454 (O103:H2) (11). Strains were stored at –70°C in 30% glycerol and were grown in Luria-Bertani (LB) agar and in LB broth at 37°C. All non-O157 STEC strains were kindly provided by the Laboratory for Food-Borne Zoonoses, Guelph, Ontario, Canada.

Cloning of LEE and non-LEE genes. The STEC O157:H7 strain EDL933 was used as the source of DNA. The desired region of chromosomal DNA was amplified by PCR, allowing for the introduction of unique restriction sites cloned into the pQE-30 plasmid (Qiagen) for 6×His-tagged proteins (Qiagen) and the pGEX-5X-1 plasmid for glutathione S-transferase (GST)-fused proteins. Ligations were completed using the Rapid DNA ligation kit as described by the manufacturer (Fermentas). Plasmids were chemically transformed into E. coli JM105 cells (pQE-30) and E. coli BL21 cells (pGEX-5X-1). Primers and restriction sites for genes cloned can be found in Table S1 in the supplemental material.

Expression and purification of His-tagged LEE and non-LEE proteins. An overnight LB culture was inoculated at 1:100 into fresh LB supplemented with ampicillin (100 µg/ml). The culture was grown at 37°C with shaking to an absorbance of 0.6 at 600 nm and induced for 3 h with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Bacteria were pelleted, and His-tagged proteins were purified with nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) under denaturing conditions using the protocol from Qiagenexpressionist (Qiagen). The purity of proteins was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie brilliant blue staining (16).

Expression and purification of GST fusion proteins. GST-fused proteins were expressed and purified as described previously (10). Briefly, a culture containing 500 ml LB, ampicillin (100 µg/ml), and chloramphenicol (50 µg/ml) was inoculated with 3 ml of an overnight culture containing the desired plasmids in BL21 cells. Bacteria were grown at 37°C with shaking to an absorbance of 0.2 at 600 nm, at which point IPTG was added at a concentration of 0.25 mM and cultures were incubated for an additional 3 h at 30°C. Bacteria were pelleted and resuspended in binding buffer (50 mM NaCl, 2.7 mM KCl, 10.15 mM NaHPO4, 1.75 mM KH2PO4, 10 mM MgCl2, 1% [vol/vol] Triton X-100, 50 µg of DNase I, 30 µg/ml phenylmethylsulfonyl fluoride [PMSF], 1 µg/ml aprotinin, 1 µg/ml pepstatin A, 10 µg/ml leupeptin [pH 7.4]), followed by sonication (three times for 30 s with a 1-s pulse and a 12-mm probe at maximum power; Vibra-Cell, Sonics & Materials Inc., Danbury, CT). GST-fused proteins were purified by adding 1 ml of a 1:1 slurry of glutathione-Sepharose 4B beads (Amersham) in phosphate-buffered saline (PBS) to 10 ml of cleared lysate. The beads then were washed four times with 15 ml of binding buffer. The purity of proteins was visualized following SDS-PAGE using Coomassie brilliant blue staining (16).

RESULTS

Immune responses against STEC O157:H7 secreted proteins determined by Western blotting. Bovine and rabbit sera raised against STEC O157:H7 T3SSPs were tested against 37 LEE-purified proteins to investigate if the pattern of reactive proteins was similar. Both rabbit and bovine sera reacted with the same proteins (Tir, EspA, EspB, EspG, and EspD), and the only difference observed was in band intensity (Fig. 1B and G). Rabbit anti-O26-, anti-O103-, anti-O111-, and anti-O157-specific sera raised against T3SSPs also were tested against the 37 LEE proteins to determine which cross-reactive serotype proteins were present in the bacterial culture (Fig. 1C to E and Tables 2 and 3). The pattern of LEE proteins by Western blotting was comparable, with Tir, EspA, EspB, and EspD reacted with sera from all serotypes. The EspF and EspG proteins were detected by the majority of the sera, and SepD and EscC were detected by at least one individual serum tested.

A total of 29 non-LEE purified STEC O157:H7 secreted proteins also were tested for reactivity with anti-rabbit O26, O103, O111, and O157 secreted proteins (Tables 2 and 3; also see Fig. S1 and S2 in the supplemental material). This pattern of recognized proteins varied, where only NleA and TccP reacted with all sera, NleE and NleH with the majority, and EspY1, NleG2-1, NleG2-2, NleI, EspR1, and NleF with at least one individual serum sample tested. Overall, O157-specific serum reacted to the least number of purified proteins (8 of 66), while O103-specific serum reacted to the most proteins (15 of 66). Rabbit preimmun
serum was used as a negative control against all proteins (Fig. 1F; also see Fig. S1F and S2F in the supplemental material), as well as an anti-6×His monoclonal antibody (Fig. 1H; also see Fig. S1H and S2H in the supplemental material).

**Immune responses against STEC O157:H7 secreted proteins determined by ELISA.** To obtain a quantitative measure of the response against STEC T3SSs, ELISAs were used to analyze the reactivity of purified STEC O157:H7 secreted proteins with rabbit sera raised to STEC O157, O26, O103, and O111 T3SSs. A total of 20 proteins were tested, including 18 proteins that were positive by Western blotting (Table 2) and 2 negative proteins (NleG6-1 and Map). Preimmune, preimmune sera; O157, rabbit anti-O157 T3SP polyclonal antibodies; O26, rabbit anti-O26 T3SP polyclonal antibodies; O103, rabbit anti-O103 T3SP polyclonal antibodies; O111, rabbit anti-O111 T3SP polyclonal antibodies. Values represent OD of greater than 0.050 after the subtraction of the values from the preimmune control sera (Fig. 2; also see Fig. S3 in the supplemental material). ELISA was completed using sera from experimentally infected cattle. A total of seven proteins gave an ELISA OD of greater than 0.050 after the subtraction of the values from the preimmune control sera (Fig. 2; also see Fig. S3 in the supplemental material). Five of the seven proteins resulted in significant reactivity when the sera from experimentally infected or vaccinated animals against T3SSs were used (Tir, EspA, EspD, EspB, and NleA).

**Reactivity of human sera from HUS patients against recombinant purified STEC O157:H7 secreted proteins.** To compare the profile of T3SSs recognized by sera from HUS patients previously infected with STEC relative to those of cattle described above, we tested the reactivity of sera from six individuals against the 66 purified STEC O157:H7 secreted proteins. Twelve proteins gave a positive ELISA reading relative to those of naive controls. Four proteins (Tir, EspD, EspA, and NleA) reacted with the majority of sera tested (Fig. 2; also see Fig. S4 in the supplemental material), while EspG, EspB, RoxF1, and EscC demonstrated significant levels of reaction to at least one individual serum sample. The highest level of reactivity was seen with the Tir protein, where all six samples demonstrated elevated readings.

**DISCUSSION**

The production and secretion of T3SSs by STEC strains is essential for colonization, as these proteins are involved in the
formation of A/E lesions, which are critical for bacterial persistence in both bovine and human hosts. Type III secreted proteins have been shown to have protective properties, since vaccination with a culture supernatant containing T3SPs significantly reduced the number of animals shedding STEC, as shown in Table 2. Summary of reactive recombinant STEC O157 T3SPs against rabbit O26-, O103-, O111-, and O157-specific sera.

| Protein | Reactivity after exposure to: |
|---------|-------------------------------|
|         | O157 | O26 | O103 | O111 |
| LEE     |       |     |      |      |
| EscC    | ++    | ++  | +    | +    |
| SepD    | +     | +   | +    | +    |
| Tir     | ++    | ++  | ++   | ++   |
| EspA    | ++    | ++  | ++   | ++   |
| EspB    | ++    | ++  | ++   | ++   |
| EspG    | +     | +   | +    | +    |

| Non-LEE |       |     |      |      |
| NleA    | ++    | ++  | ++   | ++   |
| NleE    | +     | +   | +    | +    |
| NleF    | +     | +   | +    | +    |
| NleH    | +     | +   | +    | +    |
| Niel    | +     | +   | +    | +    |
| NleG2-1 | +     | +   | +    | +    |
| NleG2-2 | +     | +   | +    | +    |
| TccP    | +     | +   | +    | +    |
| EspYl   | +     | +   | +    | +    |

* Shown are LEE and non-LEE proteins which reacted against O26-, O103-, O111-, and O157-specific sera in Western blots. A plus sign represents reactivity.

Table 3. Summary of reactive recombinant STEC O157 T3SPs against sera from O157-experimentally infected and O157-vaccinated cattle.

| Protein | Vaccinated with STEC O157:H7 TSPs | Experimentally infected with STEC O157:H7 |
|---------|----------------------------------|------------------------------------------|
| LEE     |                                  |                                          |
| Tir     | ++                               | +                                        |
| EspA    | ++                               | +                                        |
| EspD    | +                                | ++                                       |
| EspB    | +                                | ++                                       |
| EspG    | +                                |                                          |
| Non-LEE | EspM2                            | NleA                                     |
| NleA    | +                                |                                          |
| TccP    | +                                |                                          |

* Shown are LEE and non-LEE proteins which reacted against sera from O157-experimentally infected and O157-vaccinated cattle. A plus sign indicates reactivity.
Lysis leading to the contamination of the supernatant with these sera raised against T3SPs could be a result of bacterial infection of structural and regulatory proteins (SepD and EscC) and periplasmic structural proteins are not exposed on the cell external portion of the secretion apparatus. These results were therefore these were omitted from the analyses. These proteins purify three structural proteins (EscR, EscU, and EscV), and excluding intimin. However, we were unable to express and purify all proteins previously reported in humans and cattle, confirming that protein of T3SPs. These results, along with those for A/E lesions previously reported in humans and cattle, confirm that protein.

In this study, we have identified several other LEE and non-LEE proteins which also are cross-reactive with O26-, O103-, O111-, and O157-specific sera. The initial cross-reactivity observed with Tir and EspA was due to their sequence homology with other STEC serotypes. We believe that the observed cross-reactivity in this study also could be related to sequence homology among the serotypes.

The ELISA results presented in Table 1 were consistent with the Western blotting results for the majority of proteins which reacted with the STEC T3SP antisera (Tables 2 and 3). However, proteins such as NleE, which were positive for STEC O111 antisera by Western blotting, did not strongly react using an ELISA procedure. Two other proteins, NleH and EspY1, both were negative by Western blotting and positive by ELISA. These results could be linked to the greater sensitivity of the ELISA procedure or the level of protein denaturation that occurs during SDS-PAGE.

The ELISA data in Table 1 also demonstrate how STEC strains can be grouped based on reactivity and secretion profiles. For example, proteins such as Tir, EspA, EspB, and NleA all are secreted and appear to be cross-reactive with sera specific for all serotypes tested. Other proteins, such as EspF, TccP, NleG2-1, and NleG2-2, are cross-reactive with a number of non-O157 serotypes, such as STEC O103, but not with STEC O157 serum. The lack of reactivity observed with STEC O157 sera could be a result of low secretion levels of the specific proteins. However, further testing is required to confirm this possibility. For the majority of proteins which showed low reactivity with non-O157 sera, we are unable to conclude if these results are due to reduced homology, secretion levels, or the presence or absence of specific epitopes, since all proteins were expressed from STEC O157 genes. We previously showed with the Tir protein that the location of reactive epitopes differed significantly by serotype (unpublished results). To properly address this question, all proteins tested also would have to be purified from the non-O157 serotypes used.

The main reservoir for STEC is ruminants, and cattle are considered the most important source of human infection. These animals are colonized by highly virulent STEC strains without ever causing overt disease. Interestingly, STEC still are able to cause A/E lesions in cattle intestinal epithelium (3). In humans, STEC infection involving A/E lesions leads to hemorrhagic colitis, which results in complications such as HUS and TTP (7). In this study, we compared sera from experimentally infected cattle and human HUS patients against LEE and non-LEE T3SPs to determine if there were major differences in the host response. In general, the majority of immunogenic proteins were recognized by both bovine and human sera. While the bovine response against the purified proteins was fairly consistent, the magnitude of the response by the human HUS sera appeared to differ. The Tir protein gave the highest response of all tested proteins, which was not unexpected based on previous studies (26). The bulk of the immunogenic proteins were structural components involved in the secretion of T3SPs. These results, along with those for A/E lesions previously reported in humans and cattle, confirm that protein.

![Graph](FIG. 2. Antibody response of sera from STEC O157:H7 experimentally infected cattle and human sera from HUS patients against STEC O157 secreted proteins. Sixty-six purified proteins were tested, and only reactive proteins (ELISA OD higher than 0.050) were graphed. Negative proteins not shown on the graph consist of Ler, Orf2, CesA/B, Orf4, Orf5, EscT, Rorf13, GrfR, CesD, EscC, SepD, EscJ, Orf8, SepZ, Orf12, CesN, Orf16, EspH, CesF, Map, CesT, EscD, SepL, CesD2, EscF, Orf29, EspF, NleB, NleB2-1, NleC, NleE, NleG, NleH1-2, NleI, NleG2-2, NleG3, NleG5-1, NleG6-1, NleG8-2, NleG9, EspK, EspL2, EspR1, TccP, EspV, EspW, EspX2, EspX7, EspY1, EspY2, and EspY3. Single-well dilutions of sera were used for each protein. Premune cattle sera were used to calculate background values against each protein. The graphed ELISA OD represents the means plus standard deviations from samples (six samples of human HUS patients and two samples from experimentally infected cattle) which were calculated by subtracting the preimmune value from the infected cattle value. Duplicate values were averaged, and three standard deviations were calculated before subtraction. Solid grey bars, sera from experimentally infected cattle; dotted bars, sera from human from HUS patients.)
secretion is functional during the natural infection of both hosts. In addition, the immunogenic proteins reported here are found in genetic mobile elements which have been highlighted by Karmali and colleagues to be useful in the characterization of serotypes to seropathotypes A through E based on their occurrence in human disease, including outbreaks and HUS cases (11). We must note that although all preimmune antisera (human and cattle) were prescreened against STEC proteins, the preimmune antisera used in this assay may not be a truly negative control, considering that many cattle and humans have preexisting antibody to STEC proteins as a result of exposure. Based on the similarity of proteins recognized by both human and cattle sera, the potential to use measurements of serological responses to STEC proteins such as Tir, EspA, EspD, and NleA to detect or diagnose past or present infections in cattle and humans also should be considered.

The majority of purified immunogenic proteins were LEE encoded (Tir, EspB, EspD, and EspA) and have been shown to participate in colonization by STEC serotypes. Purified EspB, EspA, and Tir have been tested previously with sera from HUS patients and shown to be recognized by the host during natural infection (18). The only purified non-LEE protein that appears to be highly immunogenic is NleA. This protein has been shown previously to be involved in the modulation of virulence by the A/E pathogen Citrobacter rodentium (17). Recently, this protein also has been shown to play a role in the disruption of host intestinal tight junctions (30). However, the role of NleA in the colonization of humans and cattle remains unknown.

Interestingly, many of the secreted proteins were recognized by antibodies from experimentally infected cattle and human HUS patients even though these proteins are injected into the host cell and remain intracellular. It is possible that epitopes from a number of secreted proteins could be presented on the surface of the infected cell much like virus antigen, resulting in a cell-mediated response. However, in this study we focused on the humoral response to secreted proteins. In future studies it may be critical to also investigate the role of cell-mediated responses against the colonization and infection of STEC serotypes.

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