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Vaccination with killed whole-cells of *Escherichia coli* O157:H7 *hha* mutant emulsified with an adjuvant induced vaccine strain-specific serum antibodies and reduced *E. coli* O157:H7 fecal shedding in cattle

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ABSTRACT

Escherichia coli O157:H7 (O157) can cause from a mild diarrheal illness to hemorrhagic colitis and hemolytic uremic syndrome in humans. Cattle are the primary reservoir for O157 and fecal shedding of O157 by these animals is a major risk factor in contamination of cattle hides and carcasses at slaughter. Vaccination is an important strategy to reduce fecal shedding of O157 in cattle. In this study, we evaluated the immunogenicity and efficacy of an inactivated vaccine strain of O157 formulated with an adjuvant. This vaccine strain was deleted of the *hha* gene enabling high level expression of the locus of enterocyte effacement (LEE) encoded proteins required for O157 colonization in cattle. The inactivated vaccine strain emulsified with the adjuvant or suspended in the phosphate-buffered saline (PBS) was injected in the neck muscles of two groups of weaned calves followed by a booster three weeks later with the corresponding formulation. Animals in groups 3 and 4 were injected similarly with the adjuvant and PBS, respectively. All animals were orally inoculated three weeks post-booster vaccination with a live culture of O157. The animals vaccinated with the adjuvanted vaccine showed higher serum antibody titers to the vaccine strain and shed O157 for a shorter duration and at lower numbers compared to the animals vaccinated with the non-adjuvanted vaccine, adjuvant-only, or PBS. Western blotting of the vaccine strain lysates showed higher immunoreactivity of serum IgG in vaccinated animals to several O157-specific proteins and lipopolysaccharides (LPS). The vaccination induced IgG showed specificity to LEE-encoded proteins and outer membrane LPS as LEE and *waaL* deletion mutants, unable to produce LEE proteins and synthesize high molecular weight LPS, respectively, yielded significantly lower antibody titers compared to the parent vaccine strain. The positive reactivity of the immune serum was also observed for purified LEE-encoded proteins EspA and EspB. In conclusion, the results of this animal study showed that a two-dose regimen of an adjuvanted vaccine is capable of inducing O157-specific immune response that directly or indirectly reduced fecal shedding of O157 in cattle.

1. Introduction

Cattle are the primary reservoir for *Escherichia coli* O157:H7 (O157), which preferentially colonizes follicular tissue at the rectoanal junction (RAJ) in these animals (Naylor et al., 2003). RAJ colonization requires virulence factors secreted through a type-III secretion system (T3SS) spanning bacterial cell membranes and terminating at the bacterial cell surface as a long filament (Garmendia and Frankel, 2005; Naylor et al., 2005). The secreted EspA protein is a major structural component of this filament that has a terminal opening bounded by the secreted EspB and EspD proteins (Ide et al., 2001). The filament promotes initial bacterial-epithelial cell interactions and allow translocation of secreted proteins into epithelial cells (Gaytan et al., 2016). Bacterial outer membrane protein Intimin interacts with one of the translocated proteins, called Tir (Translocated Intimin Receptor) enabling intimate bacterial adherence to epithelial cells (Vlisidou et al., 2006). The genes for the T3SS and secreted proteins are encoded by the pathogenicity island termed the locus of enterocyte effacement or LEE (Elliott et al., 2000). We have reported previously that Hha, which has been shown to be a negative regulator of α-hemolysin expression in *E. coli*, also acts as a negative regulator of LEE and deleting *hha* increases LEE expression and O157 adherence to HEp-2 cells (Nieto et al., 1991; Sharma et al., 2012; Sharma and Zuerner, 2004).

In addition to LEE-encoded proteins, several other outer membrane

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proteins, such as OmpA (an outer membrane protein involved in transport) and non-protein *O*-antigens (major structural components of the cell wall lipopolysaccharides) have also been considered important in bacterial adherence to tissues, colonization of bovine rectoanal junction, and the host immune system evasion (Sheng et al., 2008; Torres and Kaper, 2003). The LPS biosynthesis in O157 is highly complex (Miyashita et al., 2012) and involve the attachment of an O-antigen side chain to the core-lipidA by the ligase activity of the WaaL protein (Hug and Feldman, 2011).

O157 colonization of cattle invariably results in intermittent shedding of these bacteria in animal feces (Cray and Moon et al., 1995), which is a major risk factor for the contamination of animal hides, carcasses, and downstream spreading of O157 to food products, especially ground beef (Elder et al., 2000). Cattle vaccination has been proposed as an important strategy for reducing fecal shedding in order to reduce the risk of O157 transmission to humans (Varela et al., 2013). Since colonization of the RAJ requires virulence factors secreted by the T3SS, most vaccine strategies have targeted these factors to reduce O157 colonization in cattle. For example, vaccinating cattle with culture supernatant containing virulence proteins secreted by the T3SS, in combination with an adjuvant, reduced fecal shedding of O157 in animals experimentally or naturally infected with O157 (Potter et al., 2004). Similarly, vaccinating cattle with a mixture of recombinant EspA, intimin, and Tir with or without H7 flagellin, has shown to be effective in reducing fecal shedding of O157 (McNeilly et al., 2015). Cattle vaccination with outer membrane preparations enriched for siderophore receptors and porin proteins (SRP) are effective in reducing fecal shedding of O157 (Fox et al., 2009; Thornton et al., 2009). A recent meta-analysis of all the vaccines and vaccine trials conducted to date concluded that the vaccination of cattle is about 50% effective in reducing prevalence and fecal shedding of O157 (Snedeker et al., 2012; Varela et al., 2013).

In a previous study, we demonstrated that vaccinating cattle with three-doses of a heat-inactivated *hha* deletion mutant strain of O157 shortened the duration of fecal shedding of O157 inoculated orally into these animals (Sharma et al., 2011). Considering that the three-dose vaccination is not ideal for animal handling and production, we evaluated efficacy of two-dose vaccine regimen on induction of an anti-O157 immune response and its effect on fecal shedding of O157. Since some non-O157 serotypes of Shiga toxin-producing *E. coli* (STEC) can be shed from cattle and cause human disease, we also evaluated the cross-reactivity of immune sera against non-O157 strains of STEC.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacterial strains used in this study included Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 strain NADC 6564, *E. coli* O157:H7 strain NADC 6597, a non-pathogenic *E. coli* strain NADC 479, an enteropathogenic *E. coli* O55:H7, six non-O157:H7 STEC serotypes (O26, O45, O103, O111, O121, O145), and *E. coli* TOP10 (Life Technologies, Grand Island, NY). *E. coli* O157:H7 strain 6564 is a *stx2*$^+$, curli-negative, and a spontaneous streptomycin-resistant mutant of *E. coli* O157:H7 strain 86–24 (Sharma et al., 2016; Tarr et al., 1989). *E. coli* TOP10 was used as a host for the propagation of recombinant plasmids. Bacterial strains were propagated in Luria-Bertani broth (LB) or LB containing 1.5% agar (LB-agar). Antibiotics were added to the liquid or solid media as needed (streptomycin 100 mg per liter; kanamycin 50 mg per liter). For making the whole cell vaccine, strain NADC 6597 was grown in Dulbecco's Modified Eagle's Medium containing glucose (DMEM) (Life Technologies, Grand Island, NY) to enhance the expression of T3SS and secreted proteins (Roe et al., 2007).

2.2. Recombinant DNA procedures

The vaccine strain was constructed by deleting the genes *stx2* and *hha* in strain NADC 6564 (Sharma et al., 2016). The *stx2* gene was deleted by using the phage lambda-derived Red recombination system (Datsenko and Wanner, 2000). Briefly, a 1.5 kb fragment containing the gene encoding kanamycin (*kan*) resistance, which is flanked at its 5′ and 3′ ends by FRT sequences for enabling a FLP catalyzed deletion of the *kan* resistance gene, was isolated from the pKD4 plasmid (Datsenko and Wanner, 2000) by PCR using a primer pair with the nucleotide sequences (**ggtgctgattacttcagccaaaaggaacacctgtatatgaagt**gaatcagatatctagctgactaag) and (**cacttgttacccacataccacgaatcaggttatgcctca gtcatg**aatcagatatcctcatctagttac). The bold-typed nucleotides in these primers are complementary to the nucleotides at the 5′ and 3′ ends, respectively, of *stx2* (Plunkett et al., 1999). The underlined nucleotides represent nucleotides complementary to the 5′ and 3′ ends, respectively, of the *kan* FRT fragment. The procedures for PCR amplification, purification of the amplified DNA fragments, electroporation of the purified DNA fragments in to arabinose-induced competent bacterial cells (strain NADC 6564 in the current study) containing the pKD46 plasmid, selection of *kan*-resistant isolates, removal of the *kan* gene, and confirmation of the *stx2* gene deletion have been described previously (Sharma and Bearson, 2013). The *hha* gene was then deleted in the *kan*-sensitive *stx2* deletion mutant (strain NADC 6595) by using the pSM122 plasmid and the allelic replacement procedure described in a previous study (Sharma and Zuerner, 2004). The newly constructed *stx2 hha* deletion mutant (strain NADC 6597) strain was tested for its ability to secrete higher amounts of secreted virulence proteins and produce an hyper-adherent phenotype on HEp-2 cells as described in a previous study (Sharma et al., 2012). For constructing deletion mutants lacking the LEE region (ΔLEE) or the *waaL* gene (Δ*waaL*), 1.5 kb fragments containing the *kan* FRT sequence were amplified from pKD4 as described above using primer pairs specific for LEE (**ggttacccttttcttc-gattgctcataggcagctaaatgatctt**gaatcagatatctagctgactaag and **gtttctat-tattagttttgattaataaagttttattttaagtgtg**aatcagatatcctcatctagttac) and *waaL* (**tgaaataataaccaataagttgacatcggagataagatgacct**gaatcagatatctagctgactaag and **aataaaaaggccgcattatgcagccttttttattttttacttgt** gaatcagatatcctcatctagttac) deletions. These fragments were used in generating LEE and *waaL* deletions using the procedure described above for deleting the *stx2* gene. The deletion of these regions was confirmed by analyzing DNA fragments resulting from PCR amplification of genomic DNA of these mutants with specific primers capable of priming short regions upstream (LEE region deletion confirmation primer-ggatgaagccatctaagtaatac; *waaL* region deletion confirmation primer-gaagtattgccgaagatgagtc) and downstream (LEE region deletion confirmation primer-gcatcagagataaaaggcagtg; *waaL* region deletion confirmation primer-gtcattccttcccagttccag) of the deleted region by standard agarose gel electrophoresis.

2.3. Isolation of outer membrane lipopolysaccharides of the vaccine strain

An overnight culture was diluted 1:100 in DMEM, grown for 5 h at 37 °C on a shaker (200 rpm). The bacterial cells were harvested (3000 × *g* for 15 min) and used for lipopolysaccharides (LPS) isolation using a commercial kit and according to the manufacturer's instructions (Bulldog Bio, Inc, Portsmouth, NH). The purified LPS preparation was digested with proteinase K at 60 °C for 50 min and heated for 10 min at 95 °C to remove contaminating proteins.

2.4. Generation and purification of the EspA and EspB proteins

The ORFs encoding for the EspA and EspB proteins were isolated by PCR using primers (*espA*-Forward: caggatccatggatacatcaaatgcaac/*espA*-Reverse: cagtcgacttatttaccaagggatattgc) and (*espB*-Forward: gcgggatc-catgaatactattgataatactcaag/*espB*-Reverse: gcggtcgacttacccagctaagc-gacccg), respectively, from *E. coli* O157:H7 strain EDL933 (GenBank