Immunization with H7-HCP-Tir-Intimin Significantly Reduces Colonization and Shedding of Escherichia coli O157:H7 in Goats

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Abstract

Enterohemorrhagic Escherichia coli (EHEC) O157:H7 is the causative agent of hemorrhagic colitis and hemolytic uremic syndrome in humans. However, the bacterium can colonize the intestines of ruminants without causing clinical signs. EHEC O157:H7 needs flagella (H7) and hemorrhagic coli pili (HCP) to adhere to epithelial cells. Then the bacterium uses the translocated intimin receptor (Tir) and an outer membrane adhesion (Intimin) protein to colonize hosts. This leads to the attachment and effacement of (A/E) lesions. A tetravalent recombinant vaccine (H7-HCP-Tir-Intimin) composed of immunologically important portions of H7, HCP, Tir and Intimin proteins was constructed and its efficacy was evaluated using a caprine model. The results showed that the recombinant vaccine induced strong humoral and mucosal immune responses and protected the subjects from live challenges with EHEC O157:H7 86-24 stain. After a second immunization, the average IgG titer peaked at 7.2 x 10^5. Five days after challenge, E. coli O157:H7 was no longer detectable in the feces of vaccinated goats, but naïve goats shed the bacterium throughout the course of the challenge. Cultures of intestinal tissues showed that vaccination of goats with H7-HCP-Tir-Intimin reduced the amount of intestinal colonization by EHEC O157:H7 effectively. Recombinant H7-HCP-Tir-Intimin protein is an excellent vaccine candidate. Data from the present study warrant further efficacy studies aimed at reducing EHEC O157:H7 load on farms and the contamination of carcasses by this zoonotic pathogen.

Introduction

Enterohemorrhagic Escherichia coli (EHEC) O157:H7 is a zoonotic enteric pathogen associated with hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in humans. Ruminants are the main reservoir of E. coli O157:H7 which usually colonizes the intestinal tract without causing clinical signs [1]. Infected animals can shed the bacteria in their feces, so becoming direct or indirect sources of human infections via contaminated food or water [1–2]. For this reason, EHEC O157:H7 control in ruminants merits more attention.

Reductions in the number of EHEC O157:H7 infection in cattle and in feces excreted by asymptomatic shedders can significantly decrease the risk of human exposure to this pathogen [3]. Vaccination of cattle has been proposed as a pre-harvest intervention strategy to reduce the amount of EHEC O157:H7 transmission from cattle. Inoculations of cattle with type III secreted proteins decreases fecal shedding of E. coli O157:H7 [4]. Vaccines based on siderophore receptors and porin (SRP) can reduce the burden of E. coli O157:H7 on cattle [5]. Systemic vaccination of cattle with γ-intimin C280 and EspB proteins decreases the fecal shedding of E. coli O157:H7 [6]. Immunization of cattle with a combination of purified intimin-531, EspA and translocated intimin receptor (Tir) significantly reduces shedding of E. coli O157:H7 after oral challenge [7]. Vaccination with E. coli O157 bacterial ghosts was found to provide protection in a bovine experimental model [8]. These vaccine formulations may become important tools in the control of EHEC O157:H7 transmission between animals and from animals to humans.

The versatile virulence factors contributing to E. coli O157:H7 colonization of the gastrointestinal epithelium include outer membrane proteins, type III secretion system (T3SS) proteins, flagella, and pili. These proteins are often chosen to construct recombinant vaccines. Among them, intimin (oe–1 gene) and Tir (tie–1 gene) are key colonization factors, which play significant roles in E. coli O157:H7 attachment to host epithelium [4–7]. H7 flagellin encoded by the βG gene is another interesting virulence factor. It reduces the rate of colonization but not that of overall bacterial shedding [9]. Hemorrhagic coli pili (HCP) are long bundles of type IV pili (TFP). These also contribute to bacterial colonization, virulence, and transmission of E. coli O157:H7 [10–12].
Because intimin, Tir, H7 flagellin, and HCP are critical to many of the stages of intestinal colonization by *E. coli* O157:H7, and recombinant subunit vaccines consisting of these proteins may hold the key to successful pre-harvest intervention of *E. coli* O157:H7. To test this hypothesis, a multivalent H7-HCP-Tir-Intimin protein was constructed and expressed for use as a vaccine candidate. A caprine model involving two-month-old goats was established to evaluate the effectiveness of H7-HCP-Tir-Intimin vaccine in the prevention of the colonization and spreading of *E. coli* O157:H7.

**Materials and Methods**

**Ethics Statement**

The care of laboratory animals and animal experimentation were performed in compliance with the Jiangsu Administration Guidelines for the Use of Experimental Animals. This study and all procedures were approved by the Animal Ethics Committee of Jiangsu Institute of Veterinary Medicine (SYXX20111101).

**Bacterial Strains, Plasmids and Media**

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* O157:H7 86-24 is a well-characterized Shigatoxin-producing strain. Plasmid Pcold I and pET32 were acquired from TaKaRa Corp. Bacteria are grown in Luria-Bertani (LB) broth and on LB agar (Oxoid) supplemented with 100 μg/mL of ampicillin as needed for selection of recombinant plasmids. *E. coli* O157:H7 was recovered from a freezer and cultured in brain-heart infusion (BHI) broth.

**Plasmid Construction**

Fragments of fliC, hcpA, tir, and eae were amplified by PCR from DNA isolated from *E. coli* O157:H7 86-24 strain with primers fliC-P1/fliC-P2, hcpA-P1/hcpA-P2, tir-P1/tir-P2, and eae-P1/eae-P2, respectively. The sizes of the gene fragments were 946 bp, 423 bp, 309 bp, and 811 bp, respectively. Restriction sites for appropriate restriction enzymes, ligated, and cloned into Pcold I vector. The resulting plasmid was introduced into *E. coli* BL21 by chemical transformation according to the manufacturer’s instructions (pET System Manual, Novagen Company). Plasmids pET32-fliC, pET32-hcpA, pET32-tir, and pET32-eae were produced in a previous study. The primers and restriction enzymes are described in detail in Table 2. The pColdI-fliC-hcpA-tir-eae diagram is shown in Fig. 1.

**Expression and Purification of Recombinant Protein**

An overnight culture of *E. coli* BL21 (pColdI-fliC-hcpA-tir-eae) was diluted 1:50 into a flask each containing 200 mL LB broth. When bacteria were grown to the late exponential phase (OD600 = 1.2) at 37°C, the cultures were induced with 0.5 mM IPTG and incubated for additional 12 h at 15°C. The recombinant protein was purified according to the manufacturer’s instructions (Macherey-Nagel Corp.). The bacteria were pelleted by centrifugation at 12,000 g for 5 min and resuspended in 10 ml of 1×Lysis-Equilibration-Wash buffer (LEW) buffer. The concentrated bacterial suspensions were then sonicated (60% power, 5 s on, 5 s off, 10 min) and centrifuged at 12,000 g for 30 min again. The supernatant was collected and purified using HisBind<sup>TM</sup> Resin Chromatography according to the manufacturer’s instructions (Macherey-Nagel Corp.). The expected size of recombinant His-H7-HCP-Tir-Intimin was approximately 86 kD. Similarly, recombinant Trx-H7, Trx-HCP, Trx-Tir, and Trx-Intimin proteins were expressed [14–17]. They were 51 kD, 34 kD, 30 kD, and 50 kD, respectively. The purity of these recombinant proteins was validated by SDS-PAGE and proteins were stored in −80°C (Figure 2).

**Polyclonal Antibody Preparation and Western Blotting Assay**

Specific polyclonal antibodies against His-H7-HCP-Tir-Intimin, Trx-H7, Trx-HCP, Trx-Tir, and Trx-Intimin were produced by subcutaneously injecting 4-month-old New Zealand white rabbits (2 rabbits per protein). Approximately 2 mL of recombinant vaccine containing 1 mg of purified protein was emulsified 1:1 in Freund complete adjuvant was injected at multiple sites on the animals’ necks. The rabbits received one booster injection with the same amount of antigen emulsified 1:1 with Freund’s incomplete adjuvant 21 days later. They were bled 10 days after

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**Table 1.** Bacterial strains and plasmids used in this study.

| Strain or plasmid | Description | Source |
|-------------------|-------------|--------|
| *E. coli* strain  |             |        |
| O157:H766-24     | Produce stx1 and stx2 | Current lab |
| BL21 (DE3)       | Host for expressing recombinant plasmid | Transgen |
| Vectors          |             |        |
| Pcold I          | *E. coli* expression vector | Takara |
| pET32a           | *E. coli* expression vector | Novagen |
| Recombinant plasmids |             |        |
| pET32-fliC       | pET32 carrying 538–1483 bp of fliC | Current lab |
| pET32-hcpA       | pET32 carrying 19–441 bp of hcpA | Current lab |
| pET32-tir        | pET32 carrying 775–1083 bp of tir | Current lab |
| pET32-eae        | pET32 carrying 1995–2805 bp of eae | Current lab |
| Pcold I-fliC-hcpA-tir-eae | fliC-hcpA-linker-tir-linker-eae cloned into Pcold I | Present study |

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the booster. Sera were filter (0.2 μm) sterilized and stored at −80°C until needed for a Western blotting assay.

The purified proteins were subjected to 12% SDS-PAGE and transferred to nitrocellulose membrane (Zhuayan Company, Nanjing). The membranes were blocked in 5% nonfat dry milk in Tris-Buffered Saline (TBS), pH 7.2, containing 0.1% Tween 20 (TBST) overnight at 4°C and then incubated with corresponding primary antibodies, including rabbit anti-H7, anti-HCP, anti-Tir and anti-Intimin (1:1,000 dilution) for 2 h. After three washes with 0.5% nonfat dry milk in TBST, the membranes were incubated with a 1:4,000 dilution of HRP-conjugated anti-rabbit IgG (Boster, Wuhan, China). Antigen-antibody complexes were visualized with the 3, 3′-diaminobenzidine (DAB) detection kit (Boster, Wuhan, China). Control immunoblots were performed with pre-immunization rabbit sera.

Immunization and Challenge of Goats

Twelve 2-month-old male goats were divided into two groups and housed in 4 pens with three goats per pen. Goats were raised in the Animal House of Jiangsu Institute of Veterinary Medicine. On the day before vaccination (day 0), all animals were screened to confirm that they were negative for serum antibodies against His-H7-HCP-Tir-Intimin and that they did not shed E. coli O157:H7 in their feces. Six goats were injected subcutaneously at multiple sites on their necks with 200 μL recombinant antigen (Boster, Wuhan, China). Antigen-antibody complexes were visualized with the 3, 3′-diaminobenzidine (DAB) detection kit (Boster, Wuhan, China). Control immunoblots were performed with pre-immunization rabbit sera.

Table 2. Primers.

| Primers | Sequence (5’-3’)* | Location in their ORF | Description |
|---------|-------------------|----------------------|-------------|
| fliC-p1 | ttagctagcatattacaacaa | 538–1483 nt | 5’ end of flic with Sac I site |
| fliC-p2 | ttagctagcatattacaacaa | 538–1483 nt | 5’ end of flic with Sac I site |
| hcpA-p1 | ttagctagcatattacaacaa | 538–1483 nt | 5’ end of hcpA with Sac I site |
| hcpA-p2 | ttagctagcatattacaacaa | 538–1483 nt | 5’ end of hcpA with Sac I site |
| eae-p1 | ttagctagcatattacaacaa | 538–1483 nt | 5’ end of eae with Sal I site |
| eae-p2 | ttagctagcatattacaacaa | 538–1483 nt | 5’ end of eae with Xba I site |

* : Unique restriction cleavage sites introduced in the oligonucleotides are underlined; linker sequences added to the primers are bold.

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Goat serum samples were added to the ELISA plates (100 μL/well) of bovine serum albumin (BSA) in PBST overnight at 4°C. Two-fold serially diluted co-buffer solution (Sigma) was added to the mixture for 45 min at 37°C. H7-HCP-Tir-Intimin Reduces Goat Shedding

Figure 2. The purity of Trx-H7, Trx-HCP, Trx-Tir, Trx-Intimin, and His-H7-HCP-Tir-Intimin was assayed by SDS-PAGE. Lane M, molecular mass standards; lane 1, His-H7-HCP-Tir-Intimin (88 kD); lane 2, Trx-Intimin (50 kD); lane 3, Trx-Tir (30 kD); lane 4, Trx-H7 (51 kD); lane 5, Trx-HCP (34 kD).

All the animals were euthanized 14 days after challenge using intravenous administration of 100 mg/Kg pentobarbital sodium. Then E. coli O157:H7 colonization was evaluated. Twenty-five grams of tissue from the rumen, abomasum, duodenum, jejenum, ileum, cecum, spiral colon, colon, rectum, and rectoanal junction (RAJ) of these experimental goats were collected. Tissue samples were homogenized using a stomacher and 10-fold serial dilutions were prepared. Tissue bacterial load was examined using direct culturing and IMS with O157 Dynabeads as described above in the description of the processing of feces.

**Determination of Serum IgG**

Antibody responses specific to H7-HCP-Tir-Intimin were assessed using an enzyme-linked immunosorbent assay (ELISA) as described previously [18–19]. ELISA plates (Costar, U.S.) were coated with H7-HCP-Tir-Intimin protein (2.5 μg/mL in coating buffer) overnight at 4°C. The plates were blocked with 0.1% bovine serum albumin (BSA) in PBST. Two-fold serially diluted goat serum samples were added to the ELISA plates (100 μL/well) and incubated 1.5 h at 37°C. After washing with PBS containing 0.05% Tween 20 (PBST), rabbit anti-goat IgG-HRP (1/10,000 in PBST) (KPL Corporation) was added and incubated for 45 min at 37°C. Plates were washed 3 times with PBST, TMBS substrate solution (Sigma) was added to the mixture (100 μL/well) and incubated for 5 min at room temperature. The optical density (OD) at 450 nm was determined using a Sunrise™ Absorbance Reader (Sunrise, Austria). The titer was defined as the reciprocal of the highest dilution of a serum sample producing an absorbance (OD) value above the pre-immune level. Differences in immune responses between experimental groups were analyzed using Microsoft Excel, and the t-test was used to analyze antibody responses.

Bacterial Adherence to HEp-2 Cells

For adherence experiment, HEp-2 cells were cultivated at 37°C under 5% CO₂ atmosphere in polystyrene 6-well plates (Costar, U.S.) containing glass coverslips, as previously described [20]. For the adherence assay, cell monolayers at 70% confluency were washed and cultured with DMEM (Invitrogen). A sample (10 μL of 2 x 10⁷ bacteria grown overnight in brain-heart infusion (BHI) plus NaHCO₃ (BHIN) (Zhuyan Company, Nanjing, China) at 37°C was added to wells, and the cells were infected for 3 h, washed with PBS to remove unbound bacteria. Fresh medium was added to each well and incubated for additional 3 h and washed with PBS to remove the rest of unbound bacteria. Cells were fixed with 70% methanol/PBS for 5 minutes, and stained with Giemsa for 20 minutes. The coverslips were mounted on glass slides to be observed by light microscopy.

For experiment of suppressive adherence, the procedures were similar to those described above excepting that E. coli O157:H7 was incubated with vaccination sera before infection Hep-2 cells. Briefly, E. coli O157:H7 were mixed with anti-fold diluted vaccination sera and incubated at 37°C under 5% CO₂ atmosphere for 30 min [14].

**Results**

**SDS-PAGE and Western Blotting Analysis of Recombinant Proteins**

All purified protein antigens were examined by SDS-PAGE and Coomassie blue staining. A single band of the expected molecular weight was identified for each protein sample (Figure 2). The H7-HCP-Tir-Intimin protein was detected in the supernatant fraction of lysed BL21 (Pcold I-fliC-hcpA-tir-eae), indicating the protein was soluble (Figure 3A). To determine whether each component of recombinant H7-HCP-Tir-Intimin protein was immunogenic, Western blot analyses were carried out using rabbit anti-H7, anti-HCP, anti-Tir, and anti-Intimin sera, with pre-immunization sera as a negative control. Results showed that recombinant H7-HCP-Tir-Intimin protein reacted strongly to these-mentioned sera, as evidenced by the distinct band for each corresponding antibody and the lack of reactivity to pre-immunization sera (Figure 3B). The data indicated that each component of H7-HCP-Tir-Intimin had strong immunogenicity.

**Stimulation of Immune Responses in Subcutaneously Immunized Goats**

On day 7, vaccinated goats produced an average IgG titer of 733. Specifically, 4 vaccinated goats had IgG titers of 800 and the remaining 2 goats had titers of 200 and 1600, respectively (Figure 4A). On day 21, the immunized goats had an average titer of 1.4 x 10⁶. The average IgG titer peaked after administration of booster shots (day 35) containing H7-HCP-Tir-Intimin, at
Each serum sample was then further evaluated by Western blot analysis. The present results indicate that vaccination sera interacted with recombinant H7-HCP-Tir-Intimin protein, and naïve sera did not.

In addition, secretory IgA titers specific for H7-HCP-Tir-Intimin, H7, HCP, Tir and Intimin were determined using ELISA. Two weeks after the booster shot, H7-HCP-Tir-Intimin-specific IgA was detected in the feces of 5 goats, H7-specific IgA was detected in 4 goats, and no IgA was detected in any of the unvaccinated animals. These results suggest that H7 and HCP of H7-HCP-Tir-Intimin antigen play a vital role in eliciting IgA secretion into the intestinal lumen (Figure 5).

Challenge and Excretion of E. coli O157:H7

After challenge with E. coli O157:H7, no goats developed diarrhea. Vaccinated animals shed significantly less bacteria than goats of the placebo group (P = 0.0109) (Figure 6). From day 5 on, E. coli O157:H7 was not detected in the feces of vaccinated goats. One vaccinated goat stopped shedding on day 3. In contrast, all but one of the naïve animals shed bacteria throughout the course of challenge (14 days). That goat became negative on day 10.

After euthanasia, direct fecal culture was positive for 5 naïve animals. E. coli O157:H7 carriage in feces ranged from 5.6 × 10⁴ CFU/g to 3.2 × 10⁵ CFU/g. After the enrichment and IMS, all intestinal tissues of the placebo-vaccinated animal were positive, but E. coli O157:H7 was not found in any vaccinated animals, indicating that tissue colonization was minimal or undetectable.

Bacterial Adherence to HEP-2 Cells

To further evaluate the significance of H7-HCP-Tir-Intimin antibody, each goat serum sample was detected in vitro by HEP-2 cells. For infection experiments, we propagated E. coli O157:H7 overnight in BHIN at 37°C in order to advance adherence. The light microscopy images of infected, Giemsa-stained cells showed obvious impairment of adherence of E. coli O157:H7 after incubation with vaccination sera as compared with naïve sera (Figure 7).

Discussion

In the present study, a goat model was used to evaluate the efficacy of a recombinant H7-HCP-Tir-Intimin vaccine on E. coli O157:H7 fecal shedding and host immune responses after exposure. Strong evidence showed that H7-HCP-Tir-Intimin could significantly reduce fecal shedding and provoke specific immune responses.

Because small ruminants are potential reservoirs of E. coli O157:H7, effective vaccines for these host species will have important significance to public health. In the present study, the majority of the naïve goats, all but one, shed E. coli O157:H7 throughout the entire detection period. All the tissues of their intestinal tracts were positive for E. coli O157:H7. This is consistent with previous reports stating that 8-week-old conventionally reared goats inoculated orally with E. coli O157:H7 shed bacteria until the 16th day after inoculation [21]. Animals examined on day 4 after inoculation showed multifocal AE lesions in the distal colon, rectum, and at the recto-anal junction [21]. Another study demonstrated that an 8-week-old goat kid fed E. coli O157:H7 shed the bacteria in large numbers. These numbers increased upon coincidental C. parvum infection [22]. One report showed that 20 people became ill and 1 died from E. coli O157 in organic goat’s milk cheese has been published online (http://barfblog.com/2013/09/20-sick-1-dead-from-e-coli-o157-in-raw-milk-organic-goats-milk-cheese-columnist-says-dont-overreact/). One report on an E. coli O157 outbreak in Scotland was linked to unpasteurized goat’s milk has been published online (http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId = 1307). In Greece, E. coli serogroup O157 was used to isolate of 24 strains
A study performed in Switzerland indicated that 16.3% of the evaluated goats' milk and 12.7% of ewes' milk were PCR-positive for Shiga-toxin-producing *E. coli* [24]. To date, there have been several investigations focusing on vaccine development aimed at preventing the shedding of *E. coli* O157:H7. A vaccine based on the translocon proteins EspA and EspB, and Intimin significantly reduced fecal shedding of *E. coli* O157:H7 in orally infected sheep [25]. Intranasal immunization with Stx2B-Tir-Stx1B-Zot protein led to less shedding in goats after experimental infection with *E. coli* O157:H7 [18]. The results of these studies confirmed that vaccination of small ruminants with recombinant subunit vaccines can reduce the *E. coli* O157:H7 burden in goats.

The development of multi-component vaccines has become a priority of modern vaccine research. In the current study, a tetravalent recombinant vaccine (H7-HCP-Tir-Intimin) comprising the central region of H7 (H7315), nearly full-length HcpA (HcpA141), the carboxy terminus of Intimin (Intimin270), and the exposed C-end region of Tir (Tir103) was constructed and evaluated. H7 and HCP were chosen because these proteins can block bacterial attachment in the early stage of colonization. Tir is an important T3SS protein. It acts as a receptor for Intimin. Both Tir and Intimin are the preferred choices for recombinant subunit vaccines. In cattle, vaccination with supernatant preparations containing T3SS proteins does decrease *E. coli* O157:H7 shedding in naturally infected steer [4]. A benefit to using supernatant proteins is that the preparation contains not only T3SS proteins but also flagellin, HCP, and lipopolysaccharide (LPS), which contribute to the efficacy of the vaccine. For this reason, flagellin and HCP should be selected as essential components to construct subunit vaccines.

The data from the current study showed that recombinant H7-HCP-Tir-Intimin can prevent *E. coli* O157 colonization in goats.
After the initial inoculation, serum IgG titers increased significantly. In order to improve immune responses, a booster was administered three weeks after primary immunization, the same schedule used for cattle vaccines prepared from the supernatant of bacterial cultures for achieving optimal immunity [4,26]. In the present study, the IgG titer against H7-HCP-Tir-Intimin increased 3 times after booster (7.2×10^5) compared to the titer at the primary immunization (1.4×10^5). Western blotting was carried out to assess host immune responses, and each serum from all vaccinated goats reacted to H7-HCP-Tir-Intimin, confirming the immunogenicity of the recombinant protein. Experiment of suppressive adherence to HEp-2 cells was used to evaluate the blocking ability of H7-HCP-Tir-Intimin antibody, and each serum from all vaccinated goats reduced the number of adherent bacteria to HEp-2 cells.

Fecal shedding is an important parameter of the effectiveness of recombinant E. coli O157:H7 antigens [4–7]. Our data showed there to be significantly less fecal shedding of E. coli in vaccinated goats than in control animals (P = 0.0109) [Figure 6]. E. coli O157:H7 was not detected in the feces of any vaccinated goats at any point after day 5. In contrast, most naïve goats shed bacteria throughout the course of the challenge (14 days). McNeilly et al. demonstrated that intramuscular vaccination with a combination of intimin-531, the translocon filament protein, EspA, Tir, and H7 flagellin significantly reduced shedding of E. coli O157 from experimentally infected cattle [7]. However, no study has yet shown whether goats vaccinated with this vaccine shed less E. coli O157:H7. The vaccine discussed in this paper included four immunogens, but they were expressed separately and then mixed to prepare the vaccine. This way of preparing vaccine is not economically efficient or convenient. A better vaccine design in our study is to prevent both the first adherence of E. coli O157:H7 and the consequent invasion into epithelial cells using animal immunization. For this reason, HCP and H7 were used to block

Figure 5. ELISA for H7-HCP-Tir-Intimin specific IgA titeres of feces in immunized goats. Fecal samples were taken from all goats at 0 d and 35 d (14 d post-booster) to test IgA titers. Five vaccinated goats secreted high titers of HCP- and H7-HCP-Tir-Intimin-specific IgA in feces, four animals produced certain titers of H7-specific IgA. The remaining samples, not shown here, showed no results.

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Figure 6. E. coli O157:H7 shedding in feces after subcutaneous administration in goats. Two weeks after secondary immunization, all goats in both groups were fed 10^8 CFU O157:H7 and fecal shedding was monitored every day for two weeks. The error bar shows the standard deviation and differences in outcome were assessed using a t-test (P = 0.0109).

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initial adherence and intimin and Tir were used to block invasion and colonization. In order to prepare the vaccine more easily, all individual genes were linked to the same vector of Pcold I to express one protein, in this case H7-HCP-Tir-Intimin. This plasmid vector can be used to express soluble proteins in a manner similar to their natural form [Cat. # 3360-3364, TaKaRa Corp.]. Whether the tetravalent vaccine provides the same efficacy in cattle remains to be proven. Cattle experiments must be designed to confirm its effectiveness. In summary, the tetravalent recombinant H7-HCP-Tir-Intimin vaccine was here found to prevent E. coli O157:H7 intestinal colonization and fecal shedding.

Present data also show that H7 flagella and HCP play important roles in mitigating bacterial shedding and persistence in the goat intestinal tract. Flagellin has been proposed as a vaccine candidate because of its intrinsic adjuvant activity, which is mediated through TLR5 [27-28]. Recent findings, however, suggest that vaccines against flagellated pathogens should avoid inducing antibodies (Abs) against TLR5 because antibody production may help flagellated bacteria evade host clearance by reducing the host's ability to mount an innate immune response [28-30]. The NH2- and COOH-terminal regions of flagellin are involved in interactions with TLR5 and are well conserved among various Gram-negative bacteria [30]. To circumvent this problem, the regions responsible for interacting with TLR5 were removed during the construction of the H7-HCP-Tir-Intimin tetra-protein. The results proved that H7-HCP-Tir-Intimin could prevent bacterial colonization. In contrast, a previous study showed that the production of specific IgG and IgA and impaired E. coli O157:H7 colonization in the mouse gut [15]. Taken together, the central region of H7 flagellin and full-length HCP could be important functional components of the recombinant tetra unit protein during blockage of bacterial colonization.

It has been reported that culture conditions play an important role in the ability of EHEC O157:H7 to adhere to cultured cells and to epithelial cells in the pig terminal ileum [31]. Also, the maturation of a functional TTSS is accelerated in EHEC O157:H7 by restricting the concentration of oxygen [32]. Bicarbonate ions can activate expression of LEE and certain non-LEE encoded genes, resulting in greater adherence of EHEC O157:H7 in vitro and increased shedding from 14-week-old pigs [33-34]. To ensure the success of goat challenge, E. coli O157:H7 was cultured in brain-heart infusion (BHI) plus NaHCO3 (BHIH) with gentle shaking. There were more bacteria adhering to HEp-2 under these conditions than in LB with vigorous shaking (data not shown). Collectively, the use of cultural conditions mimicking in vivo environment is essential to the development of a successful animal model for the evaluation of vaccines.

In conclusion, vaccination of goats with recombinant H7-HCP-Tir-Intimin protein reduced fecal shedding of EHEC O157:H7 very effectively. The recombinant protein can be easily produced in large quantities and the production can be standardized for consistent performance. Data from the current investigation warrants further clinical trials aimed at reducing bacterial load on farms and mitigating the contamination of carcasses by EHEC O157:H7.

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**Author Contributions**

Conceived and designed the experiments: XZ KH. Performed the experiments: XZ ZY. Analyzed the data: XZ. Contributed reagents/materials/analysis tools: KH. Wrote the paper: XZ SZ.

**References**

1. Ferens WA, Hovde CJ (2011) Escherichia coli O157:H7: animal reservoir and sources of human infection. Foodborne Pathog Dis 8(4): 465–467.
2. Keskinen LA, Annous BA (2011) Efficacy of adding detersants to sanitizer solutions for inactivation of Escherichia coli O157:H7 on Romaine lettuce. Int J Food Microbiol 147(3): 157–61.
3. Rich AR, Jepson AN, Luebbe MK, Erickson GE, Klopfenstein TJ, et al. (2010) Vaccination to reduce the prevalence of Escherichia coli O157:H7 in feedlot cattle fed wet feed diets plus soluble, Nebraska Beef Report. 94–95.
4. Potter AA, Klassinsky S, Li Y, Fery E, Townsend H, et al. (2004) Decreased shedding of Escherichia coli O157:H7 by cattle following vaccination with type III secreted proteins. Vaccine 22, 362–369.
5. Daniel UT, Guy HL, Ashley BT, Kelly FL, Daryl AE, 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(7): 871–877.
6. Viria DA, Larzabal M, Garbaccio S, Gammella M, Rahinovitz BC, et al. (2011) Reduced fecal shedding of Escherichia coli O157:H7 in cattle following systemic vaccination with y-interferon C289 and EspB proteins. Vaccine 29: 3962–3968.
7. McNeilly TN, Mitchell MC, Rosser T, McAuer S, Low JC, 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.
8. Viria DA, Larzabal M, Mayre UC, Garbaccio S, Gammella M, et al. (2012) A systemic vaccine based on Escherichia coli O157:H7 bacterial ghosts (BGs) reduces the excretion of E. coli O157:H7 in calves. Vet Immunopathol 146: 169–176.
9. McNeilly TN, Naylor SW, Mahajan A, Mitchell MC, McAuer S, et al. (2008) Escherichia coli O157:H7 colonization in cattle following systemic and mucosal immunization with purified H7 flagellin. Infect Immun 76, 2594–2602.
10. Xicotencatl-Cortes J, Monteiro-Neto V, Lealma MA, Jordan DM, Francetic O, et al. (2007) Intestinal adherence associated with type IV pili of enterohemorrhagic Escherichia coli O157:H7. J Clin Invest 117: 3319–3329.
11. Xicotencatl-Cortes J, Monteiro-Neto V, Lealma MA, Jordan DM, Francetic O, et al. (2009) the type 4 Pili of Enterohemorrhagic Escherichia coli O157:H7 are multipurpose structures with pathogenic attributes. J Bacteriol 191(1): 411–422.
12. Maria AL, Sara AO, Ariadnna C, Luz M, Rocha-Ramırez, et al. (2011) The hemorrhagic coli plus (HCP) of Escherichia coli O157:H7 is an inducer of promflammatory cytokine secretion in intestinal epithelial cells. PLoS one 29(22): 3923–3929.
13. Amani J, Moussavi SL, Rafati S, Sahmanian AH (2009) In silico analysis of chimeric espA, eae and tir fragments of Escherichia coli O157:H7 for oral immunogenic applications. Theor Biot Med Model 6: 28.
14. Zhang XH, He KW, Maoy AH, Zhou JM, Yu ZY, et al. (2010) Cloning, expression, biological activities of recombinant Tir gene from Enterohemorrhagic Escherichia coli O157:H7. Sciencia Agricultura Sinica 43(12): 2070–2577.
15. Zhang XH, He KW, Ye Q, Ni YX, Wen LB, et al. (2012) Immunoprotection of recombinant hcpA protein from enterohemorrhagic Escherichia coli O157:H7 in mice, Chin J Zoonoses 1065–1071.
16. Zhang XH, He KW, Zhao PD, Luan XF, Ye Q, et al. (2011) Clone and expression of variable fliC gene segment from enterohemorrhagic Escherichia coli O157:H7 and its immunogenicity. Jiangsu J Agric Sci 27(5): 1021–1025.

17. Zhao PD, Zhang XH, He KW, Jiang P, Lu WC, et al. (2011) Immunoprotection of Intimin and Tir-Tccp recombination protein of EHEC O157:H7 to BALB/C mice. Jiangsu J Agric Sci 27(6): 1300–1304.

18. Zhang XH, He KW, Ye Q, Yu ZY (2012) Intranasal immunization with Stx2B-Tir-Stx1B-Zot protein leads to decreased shedding in goats after experimental infection with Escherichia coli O157:H7. Vet. Rec 170(7): 178.

19. Zhang XH, He KW, Lu WC, Zhao PD (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.

20. Giron JA, Torres AG, Freer E, Kaper JB (2002) The flagella of enteropathogenic Escherichia coli mediate adherence to epithelial cells. Mol. Microbiol. 44: 361–379.

21. La Ragione RM, Ahmed NM, Weyer U, Johnson L, Pearson GR, et al. (2005) Colonization of 8-week-old conventionally reared goats by Escherichia coli O157:H7 after oral inoculation. J Med Microbiol 54: 485–492.

22. La Ragione RM, Best A, Clifford D, Weyer U, Johnson L, et al. (2006) Influence of colostrum deprivation and concurrent Cryptosporidium parvum parvum infection on the colonization and persistence of Escherichia coli O157:H7 in young lambs. J Med Microbiol 55, 819–828.

23. Solomakos N, Govaris A, Angelidis AS, Pournaras S, Burriel AR, et al. (2009) Occurrence, virulence genes and antibiotic resistance of Escherichia coli O157 isolated from raw bovine, caprine and ovine milk in Greece. Food Microbiol 26, 865–871.

24. Muehlherr JE, Zweifel C, Corti S, Blanco JE, Stephan R (2003) Microbiological Quality of Raw Goat’s and Ewe’s Bulk-Tank Milk in Switzerland. J Dairy Sci 86: 3549–3556.

25. Atef YM, Godderis BM, Vanrompay DC (2011) Immunization of sheep with a combination of intimin, EspA and EspB decreases Escherichia coli O157:H7 shedding. Vet Immun Immunol 140, 42–46.

26. Mosley RA, Smith DR, Luebbe M, Erickson GE (2009) Escherichia coli O157:H7 vaccine effect in feedlot cattle. Foodborne Pathog Dis 6(7): 879–884.

27. McNeilly TN, Mitchell MG, Rosser T, McAteer S, Low JC, et al. (2010) IgA and IgG antibody responses following systemic immunization of cattle with native H7 flagellin differ in epitope recognition and capacity to neutralize TLR5 signalling. Vaccine 28, 1412–1421.

28. Sukumar S, Fumihiko T, Tomoko M, Nao J, Kouji K, et al. (2007) Blocking of the TLR5 activation domain hampers protective potential of flagellin DNA vaccine. J Immunol 17: 1147–1154.

29. Neville LF, Barnea Y, Hammer-Munz O, Gur E, Kuzmenko B, et al. (2005) Antibodies raised against N-terminal pseudomonas aeruginosa flagellin prevent mortality in lethal murine models of infection. Inter J Mol Med 16: 165–171.

30. Anderssen-Nissen E, Smith KD, Bonneau R, Strong RK, Aderem A (2007) A conserved surface on Toll-like receptor 5 recognizes bacterial flagellin. J Exp Med 204: 393–403.

31. Yin XH, Peng YN, Wheatcroft R, Chambers J, Gong J, et al. (2011) Adherence of Escherichia coli O157:H7 to epithelial cells in vitro and in pig gut loops is affected by bacterial culture conditions. the Can J Vet Res 75: 81–88.

32. Ando H, Abe H, Sugimoto N, Tohe T (2007) Maturation of functional type III secretion machinery by activation of anaerobic respiration in enterohaemorrhagic Escherichia coli. Microbiol 153(2): 464–473.

33. Abe H, Tatsumo I, Tohe T, Okutani A, Sasakawa C (2002) Bicarbonate ion stimulates the expression of locus of enterocyte effacementencoded genes in enterohemorrhagic Escherichia coli O157:H7. Infect Immun 70: 3500–3509.

34. Best A, La Ragione RM, Clifford D, Cooley WA, Sayers AR, et al. (2006) A comparison of Shiga-toxin negative Escherichia coli O157 aflagellate and intimin deficient mutants in porcine in vitro and in vivo models of infection. Vet Microbiol 113: 63–72.