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Protective efficacy of a novel multivalent vaccine in the prevention of diarrhea induced by enterotoxigenic *Escherichia coli* in a murine model

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ABSTRACT

Background: Enterotoxigenic *Escherichia coli* (ETEC) infection is a primary cause of livestock diarrhea. Therefore, effective vaccines are needed to reduce the incidence of ETEC infection.
Objectives: Our study aimed to develop a multivalent ETEC vaccine targeting major virulence factors of ETEC, including enterotoxins and fimbriae.
Methods: SLS (STa-LTB-STb) recombinant enterotoxin and fimbriae proteins (F4, F5, F6, F18, and F41) were prepared to develop a multivalent vaccine. A total of 65 mice were immunized subcutaneously by vaccines and phosphate-buffered saline (PBS). The levels of specific immunoglobulin G (IgG) and pro-inflammatory cytokines were determined at 0, 7, 14 and 21 days post-vaccination (dpv). A challenge test with a lethal dose of ETEC was performed, and the survival rate of the mice in each group was recorded. Feces and intestine washes were collected to measure the concentrations of secretory immunoglobulin A (sIgA).
Results: Anti-SLS and anti-fimbriae-specific IgG in serums of antigen-vaccinated mice were significantly higher than those of the control group. Immunization with the SLS enterotoxin and multivalent vaccine increased interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) concentrations. Compared to diarrheal symptoms and 100% death of mice in the control group, mice inoculated with the multivalent vaccine showed an 80% survival rate without any symptom of diarrhea, while SLS and fimbriae vaccinated groups showed 60 and 70% survival rates, respectively.
Conclusions: Both SLS and fimbriae proteins can serve as vaccine antigens, and the combination of these two antigens can elicit stronger immune responses. The results suggest that the multivalent vaccine can be successfully used for preventing ETEC in important livestock.

Keywords: Enterotoxigenic *Escherichia coli*; Mice; Immune response; Small intestine; Vaccine

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is the most common cause of diarrhea in farm animals worldwide [1]. In neonatal and recently weaned piglets, ETEC-associated diarrhea results in morbidity and mortality and is considered one of the most economically important diseases in swine husbandry [2,3]. ETEC strains infect animals and cause diarrhea by producing various fimbriae and enterotoxins. When the bacterium reaches the small intestine, it

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Conflict of Interest

The authors declare no conflicts of interest.

Authors Contributions

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attaches to the intestinal epithelium via one or more fimbriae and then releases one or more enterotoxin(s), resulting in watery diarrhea [4].

ETEC strains can express a wide range of fimbriae encoded by plasmids or chromosomes to promote bacterial colonization [5]. ETEC strains with fimbriae F5, F6 and F41 cause neonatal diarrhea, while F18 strain generally causes post-weaning diarrhea in piglets. F4 strains are associated with diarrhea in both neonates and post-weaning piglets [6,7]. After colonization, ETEC strains release plasmid-encoded heat-labile (LT) or heat-stable (ST) enterotoxins, inducing severe secretory diarrhea by disrupting the water and electrolyte balance in the intestine. LT enterotoxin consists of a single A subunit and five B subunits [8]. It induces diarrhea and promotes the adhesion of ETEC [9]. ST enterotoxin is a family of peptides composed of less than 20 amino acids and exhibits poor immunogenicity [10]. There are two types of enterotoxins, including STa and STb. STa can be found in ETEC isolates from animals and humans, while STb is primarily found in animal-borne ETEC isolates, especially in post-weaning piglets [11].

Vaccination is considered the most effective way to prevent ETEC-associated diarrhea [12]. A single vaccine containing both enterotoxins and fimbriae proteins is highly desirable and will enhance the cost-effectiveness of the vaccine. We previously constructed a trivalent protein by fusing STa, LTB and STb enterotoxins (SLS). Animal immunization experiments demonstrated that SLS fusion protein could prevent ETEC-associated diarrhea [13]. In this study, SLS protein obtained from previously constructed recombinant *E. coli*, and newly extracted F4, F5, F6, F18 and F41 fimbriae proteins were mixed to develop a novel multivalent ETEC vaccine. Then, the immunogenicity and protective efficacy of the novel vaccine were investigated in a murine model.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The ETEC F4 strain C83902 (serotype O8:K87), F5 strain C83286 (serotype O38: K99:F41), F6 strain C83915 (serotype O9:K103) and F41 strain C83287 (serotype O101:F41) were obtained from the China Veterinary Culture Collection Center (China), and F18 2134 strain (serotype O157:H19) was kindly provided by Professor Guoqiang Zhu (Yangzhou University, Yangzhou, China). ETEC strains were grown in Luria-Bertani (LB) medium and modified Minca Broth (Hopebio, China) for bacterial propagation and fimbriae extraction, respectively [14].

Production and purification of recombinant SLS protein and fimbriae proteins

The production of SLS fusion protein was carried out as described previously [12]. The recombinant SLS protein was purified by Ni-NTA chromatographic column (Sangon, China) according to the manufacturer's instructions. The purified SLS antigen was concentrated to 2 mg/mL and stored at −20°C until further use.

The selected ETEC strains were cultured in modified Minca Broth (Hopebio, China) for 18 h, and then the bacterial cells were observed by transmission electron microscope (JEOL, Japan) (Model/JEM-2100) to determine the expression of fimbriae. The ETEC strains were cultured for 18 h at 37°C, centrifuged at 4,000 g for 15 min at 4°C to collect the precipitates and then resuspended with phosphate-buffered saline (PBS). The suspension was kept at 62°C for 30 min, oscillated for 10 min and centrifuged at 4°C for 15 min. The supernatant was filtered

through a membrane filter (0.22 μm filter), and the filtrate was precipitated with ammonium sulfate (60%) and kept overnight at 4°C. All fimbriae proteins were obtained as sediments after settlement and dissolved with PBS. Protein concentrations of SLS and fimbriae were measured by the BCA protein assay kit (Solarbio, Beijing, China), and the measurement was carried out according to the manufacturer's instructions. The antigens were then concentrated to 2 mg/mL and stored at −20°C [15]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to identify the extracted fimbriae proteins (**Supplementary Fig. 1**). The fimbriae proteins were mixed in equal volumes as fimbriae antigens, and the final protein concentration was 2 mg/mL.

Animals and immunization

Four-week-old, specific-pathogen-free (SPF) female Kunming (KM) mice (15–20 g) were purchased from the Experimental Animal Centre of Dalian Medical University (Dalian, China) and kept under controlled light-dark cycle (12-h light/dark cycle), humidity (50 ± 10%) and temperature (22 ± 2°C). The mice were given *ad libitum* access to food and sterilized water. The mice were acclimatized for 3 days before the start of the experiment [16]. All animal experiments were done in accordance with the recommendations of the Local Institutional Animal Care and National Act on the use of experimental animals (CSE20201109, China).

A total of 65 mice were randomly divided into five groups (n = 13 per group) and subcutaneously administered with vaccines consisting of specific antigens (three groups), adjuvant (control) and PBS (negative control), respectively (**Table 1**). The mice were injected with three prepared vaccines. The multivalent vaccine (Vm) was made by mixing SLS fusion antigen with fimbriae antigens in equal volumes. Meanwhile, two experimental vaccines were prepared separately with SLS antigen (Vs) and fimbriae antigens (Vf), respectively. The mice of the Va group were injected by the Freund's adjuvant (Sigma-Aldrich, USA) mixed with sterilized PBS to evaluate the influence of the adjuvant on animals. PBS CON group (injected with sterilized PBS) was regarded as a negative control. Each dose contained 0.3 mL of the vaccine and was performed with a sterile syringe. Mice in the PBS CON group were also administered with the same volume of PBS.

A schematic diagram of the vaccination program is illustrated in **Fig. 1**. On the day of immunization, the living conditions of mice were examined. Serum samples were collected before each vaccination, and the same procedure was performed one week after each immunization (days post-vaccination [dpv] 7 and 21). Body weight, daily food intake and water consumption of the mice were monitored during immunization. Blood was collected from the tail vein at dpv 0, 7, 14 and 21. The serum samples were collected by centrifugation of the clotted blood at 5,000 rpm for 10 min at 4°C and stored at −80°C for further analysis.

Table 1. Animal immunization groups and vaccine component description in one dose

Groups	Antigen type		Adjuvant* (mL)	Sterilized PBS (mL)
	SLS antigen (mg)	Mixed fimbriae antigens (mg)		
Vm	0.15	0.15	0.15	
Vs	0.3		0.15	
Vf		0.3	0.15	
Va			0.15	0.15
PBS CON				0.3

SLS, STa, LTB and STb; PBS, phosphate-buffered saline.
*The Freund's adjuvants were added to the vaccines to increase the immunogenicity of the antigens. The experimental animals were immunized twice with Freund's complete adjuvant for the primary immunized vaccine and Freund's incomplete adjuvant for the secondary immunized vaccine.

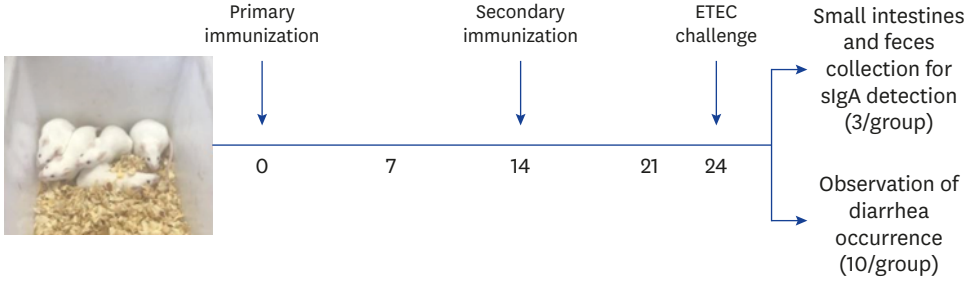

Fig. 1. Immunization with different vaccines and ETEC challenge in mice. The first vaccination date was set as 0 d, followed by the boost vaccination after two weeks (14 d). Ten days after the secondary immunization (24 d), the mice in each group were challenged with a lethal dose of ETEC.
ETEC, enterotoxigenic *Escherichia coli*; sIgA, secretory immunoglobulin A.

Challenge test

The five ETEC strains were used in inhibition tests by making a cross with broth culture on MacConkey Agar plates for every two strains using sterilized cotton swabs. All the mice were challenged at dpv 24. For the challenge test, the five strains used to prepare vaccines were mixed in equal proportions, and the total concentration of bacteria was 1×10^{10} colony forming unit (CFU)/mL. The mice were challenged orally three times at an interval of 6 h, with 1 mL of the mixed bacterial culture each time. Then, ten mice from each group were randomly selected to monitor morbidity for one week after the challenge test. The remaining three mice from each group were sacrificed for collecting samples, including small intestines and feces, after 12 h of the challenge test. The fecal samples were collected from the rectum after dissection as described previously [17].

Mouse serum specific antibody detection by enzyme-linked immunosorbent assay (ELISA)

Serum samples were examined for antigen-specific immunoglobulin G (IgG) by ELISA. ELISA was performed according to a previous study [18]. Briefly, 96-well plates were coated with SLS and fimbriae proteins (F4, F5, F6, F18 and F41), kept overnight at 4°C, washed with a washing solution containing 0.05% Tween 20 in PBS and then blocked with block buffer (PBS containing 0.1% Bovine Serum Albumin) at room temperature for 2 h. Following the washing step with a washing buffer (PBS containing 0.05% Tween 20), serially diluted serum samples were added to the plates. IgG was detected with a Goat Anti-Mouse IgG/HRP (Solarbio, China). ELISA plates were developed with TMB Single-Component Substrate solution (Solarbio) and stop solution (2 M H_2SO_4). After adding the stop solution, the plates were read at 450 nm optical density (OD450).

Measurement of cytokine interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) in serum samples

The concentrations of *IL-1β and TNF-α* were detected using commercial ELISA kits (Solarbio) following the manufacturers' instructions. In addition, OD450 was measured on a microplate reader.

Determination of the concentrations of intestinal mucosal and fecal secretory immunoglobulin A (sIgA)

The intestinal washes were collected according to a method described previously [19]. Fecal samples were suspended as 100 mg/mL in homogenization buffer (0.01 M PBS containing 0.05% Tween 20). Subsequently, the homogenates were centrifuged at 5,000 g for 10 min

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at 4°C, and the supernatants were collected for testing. The mucosal and fecal sIgAs were determined with Mouse sIgA ELISA Kit (Sangon) according to the manufacturer's protocol.

Pathophysiological observations

Ten of the challenged mice were observed for physical activity after 7 days of the challenge test. All dead mice from each group were dissected within 1 h of death. All living mice were euthanized after 7 days of the challenge test, and the small intestines were collected. The middle portions of the duodenum, jejunum and ileum were subjected to histopathological analysis. The intestine contents were washed with sterilized PBS before fixing the intestinal samples in 10% neutral-buffered formalin for 12–24 h, dehydrated with ethanol and embedded in paraffin. The samples were stained with hematoxylin and eosin (H&E) and viewed by a light microscope. A grading system of the mucosal damage was used to quantify the histological changes with some modifications [20]. Briefly, histopathology scores were graded from 0 to 3 (grade 0, normal intestinal morphology; grade 1, mild damage. A small number of epithelial cells are denuded, but the villi structures are intact basically; grade 2, moderate damage. Massive epithelial cells lift down the sides of the villi, and some tips may be denuded; grade 3, severe damage, digestion and disintegration of mucosal villi, extensive loss of crypt structures).

Statistical analysis

The results were expressed as mean ± SD and analyzed with GraphPad Prism 8.0 program (GraphPad Software Inc., USA). The statistical significance for the Kaplan-Meier survival curve was calculated by the Mantel-Cox test. One-way analysis of variance (ANOVA) was used to record the significant differences between the groups, and means were compared using Tukey's multiple comparisons test. The value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Changes in body weight, daily feed intake and water consumption during immunization

In order to evaluate the toxicity of the SLS enterotoxin and fimbriae proteins as antigens, the body conditions of mice were checked. No mice died or showed adverse reactions after vaccination, and there were no statistical differences in body weight and mean daily weight gain between the groups (**Fig. 2A and B**). Also, no significant differences were observed among the groups in terms of food intake and water consumption (**Fig. 2C and D**).

Effects of the SLS and fimbriae antigens on specific IgG responses

The levels of serum-specific antibodies against SLS enterotoxin and five fimbriae proteins are presented in **Fig. 3**. Mice immunized with the prepared vaccines containing SLS and fimbriae antigens exhibited strong humoral immune responses, yielding significantly higher titers of specific IgG than the control group during the immunization. The F4, F5 and F18 antibody titers of Vf and Vm groups were increased after the second dose, but the differences in the levels measured at dpv 14 were not statistically significant ($p > 0.05$). F6 antibody level of the Vf group was significantly enhanced after the second dose ($p < 0.05$). The F41 and SLS antibody titers of Vf, Vs and Vm groups were not increased after a booster dose but remained stable before the challenge test.

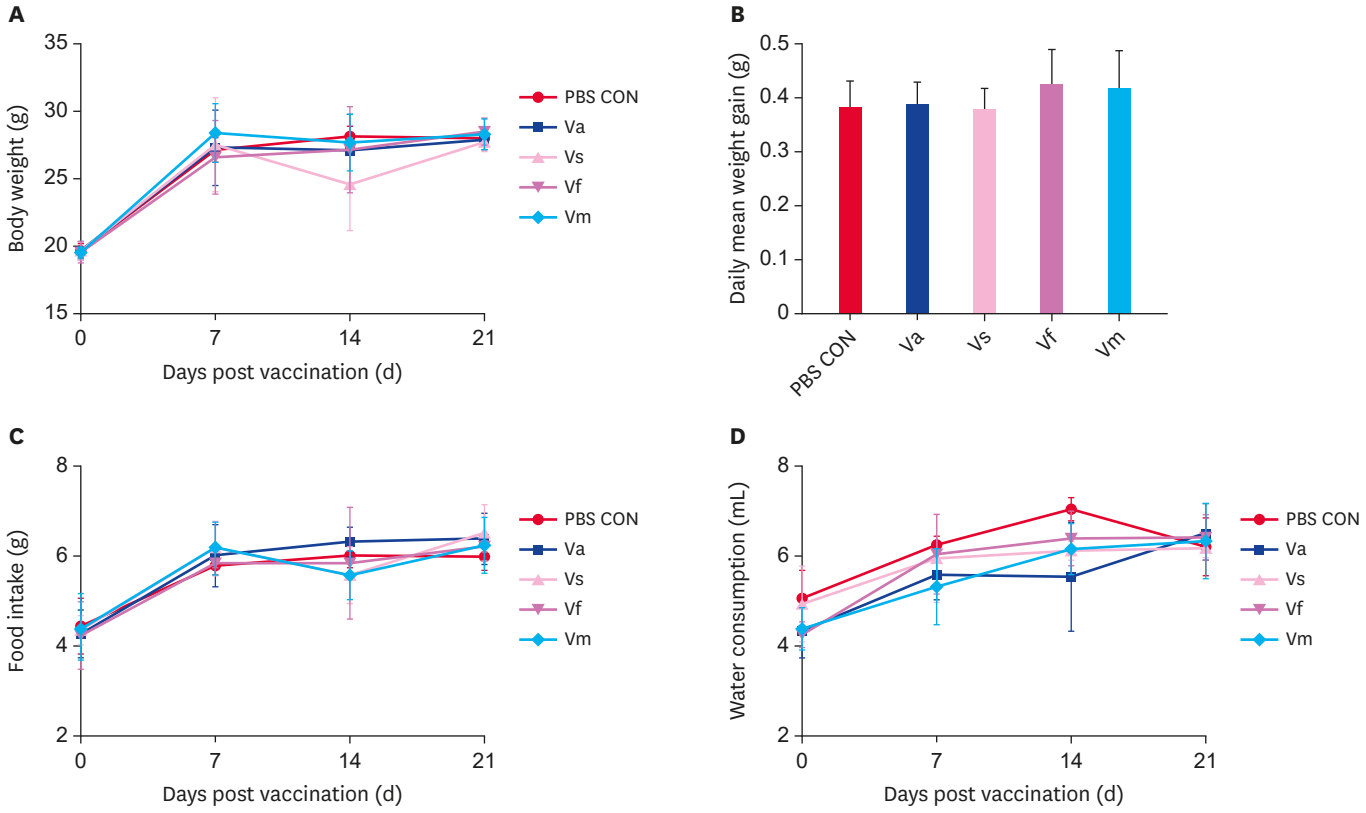

Fig. 2. Body weight, food and water consumption of mice during vaccination. Food intake and water consumption were measured in each group of mice. PBS CON was regarded as the control group. The mice in the Va group were injected with adjuvants. The mice in Vs and Vf groups were inoculated with the SLS and fimbriae antigens, respectively. The Vm group was immunized with the multivalent vaccine. (A) Changes in body weight; (B) Mean weight gain per day; (C) Changes in food intake; Water consumption (D). Data are expressed as mean ± SD.
PBS, phosphate-buffered saline; SLS, STa, LTB and STb.

Effects of inoculation with the novel vaccines on cytokine responses

The immunization with the antigens strongly induced the secretion of pro-inflammatory cytokines (IL-1β and TNF-α) (**Fig. 4A and B**). The IL-1β concentration of the Vs group was significantly higher than that of the PBS group at dpv 14 and 21 ($p < 0.05$). After the secondary immunization, the concentration of IL-1β in the Vm group was significantly higher than that in the control group ($p < 0.05$). The concentration of TNF-α in the Vs group was significantly higher than that in the other groups after the second dose ($p < 0.05$).

Mucosal immune responses of the challenged mice

The levels of total sIgA in the fecal samples and intestinal washes were determined (**Fig. 5A and B**). After infection with ETEC, the concentrations of sIgA in the feces of PBS and Va control groups were lower than those of antigen-injected groups ($p < 0.05$). Meanwhile, the titers of total sIgA antibody in the intestinal washes from mice in Vf and Vm groups were significantly higher than those from non-antigen vaccinated and normal (untreated) mice ($p < 0.05$). The titer of sIgA in the Vs group was higher than that in the normal mice, but no significant difference was observed.

Vaccines provided protective immunity to mice against ETEC challenge

There was no antagonism between the 5 ETEC strains in the strain inhibition test. In this study, an inoculum corresponding to 100 × LD_{50} for mixed ETEC strains (1×10^9 CFU) was

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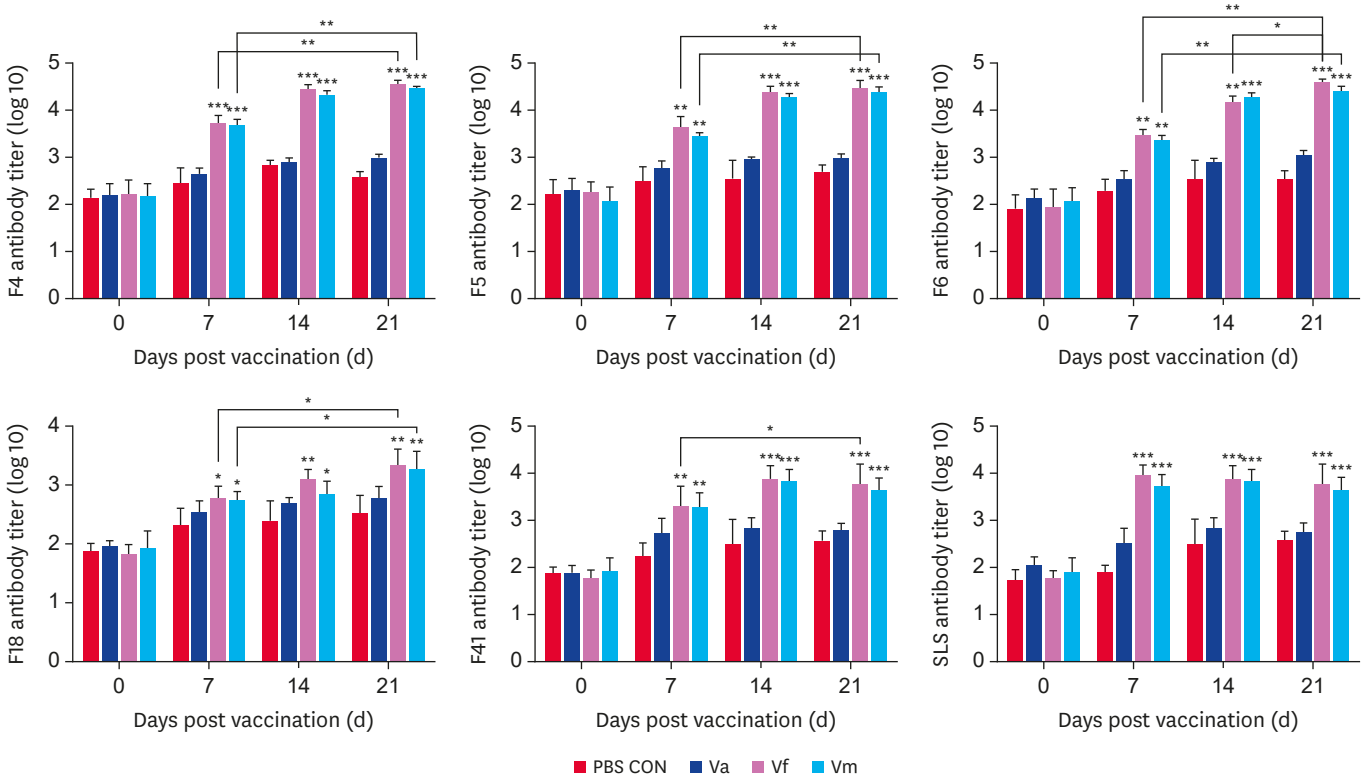

Fig. 3. Detection of specific anti-SLS and anti-fimbriae immunoglobulin G in serum samples. Vf and Vm groups were compared with Va and PBS control groups to investigate the specific antibody levels of each fimbriae antigen. The titer of anti-SLS antibody was compared among Vs, Vm, Va and PBS groups to evaluate immune responses induced by recombinant SLS protein. Data are expressed as mean ± SD. The symbols of '*', '**' and '***' indicate significantly different values from the PBS control group at the $p < 0.05$, $p < 0.01$ and $p < 0.001$ levels, respectively.
PBS, phosphate-buffered saline; SLS, STa, LTB and STb.

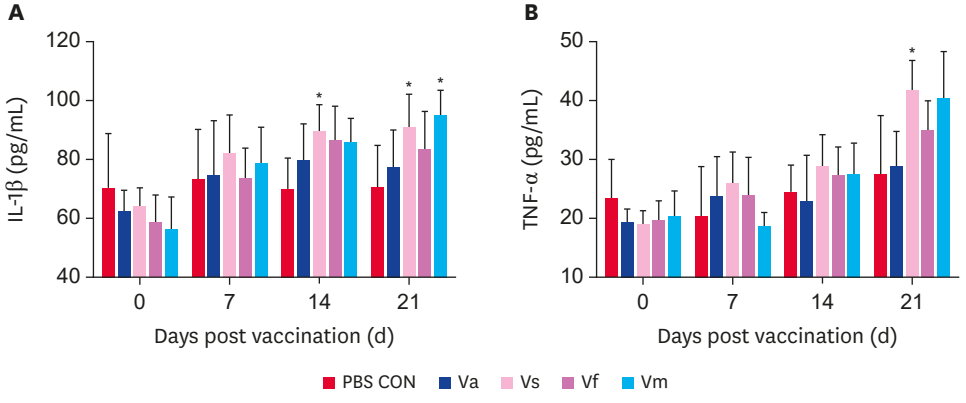

Fig. 4. Variation trends of interleukin-1β and tumor necrosis factor-α cytokines in the serum of mice. Data are expressed as mean ± SD. The symbols of '*'and '**' indicate significantly different values from the PBS control group at the $p < 0.05$ and $p < 0.01$ levels, respectively.
PBS, phosphate-buffered saline.

used for the challenge test. The survival rate of mice vaccinated with the multivalent vaccine was 80% after the ETEC challenge test, and the mice administrated with SLS and fimbriae antigens showed 60% and 70% survival rates, respectively (**Fig. 6**). In contrast, all the mice died in PBS and Va groups within 2 days after the challenge test.

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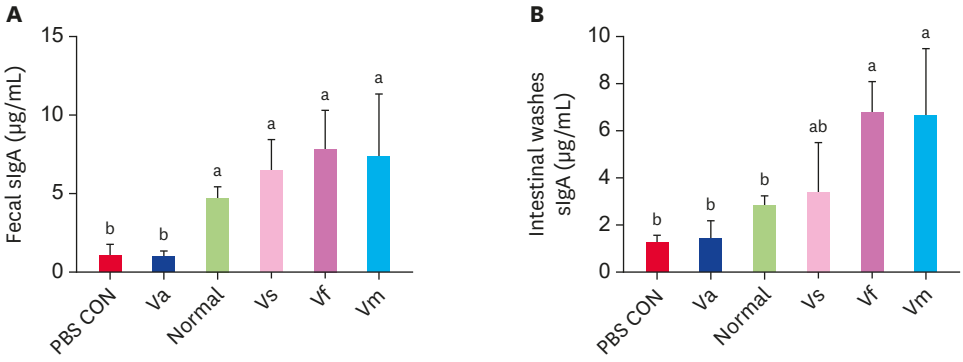

Fig. 5. The level of total sIgA in feces and intestine washes. The randomly selected mice in different groups were detected for sIgA concentration in fecal (A) and intestinal wash (B) samples. The sIgA concentration of normal (untreated) mice was detected to compare the effects of antigens on the intestinal mucosal immune system. Data are expressed as mean ± SD. Letters above the columns indicate significantly different values from each other at the $p < 0.05$ level, as determined by the unpaired *t*-test.
PBS, phosphate-buffered saline; sIgA, secretory immunoglobulin A.

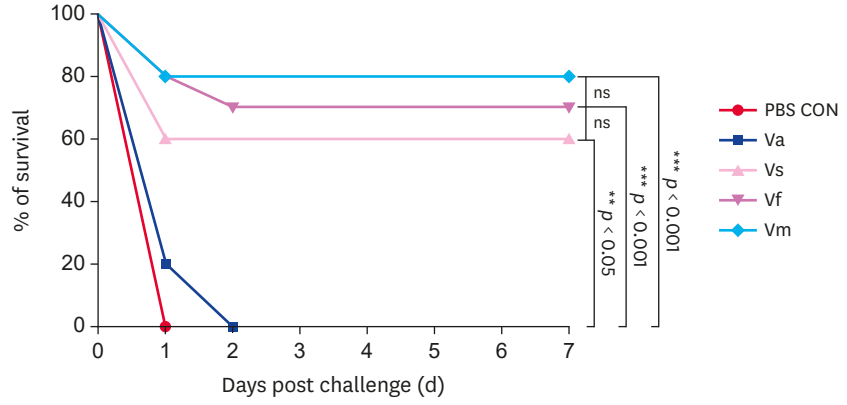

Fig. 6. Survival curves of the five groups of mice after challenge with mixed enterotoxigenic *Escherichia coli* strains. Mice in three experimental groups were immunized with different vaccines and compared with control mice treated with PBS or adjuvant alone. The survival rates on each day within 7 d after the challenge test are shown.
PBS, phosphate-buffered saline.
p values are indicated for each comparison (**$p < 0.01$; ***$p < 0.001$).

Pathophysiological responses

Intestine morphology of mice

The comparisons of small intestine morphology are shown in **Fig. 7**. Intestinal fluid accumulation and damaged intestines indicated that diarrhea was caused by ETEC strains. Intestinal fluid accumulation was not observed in Vs, Vf and Vm groups after the challenge test. Compared to the healthy mice, Va and PBS groups exhibited intestinal fluid accumulation, accompanied by intestinal ulcers and bleeding symptoms (red arrow).

Histopathological analysis

Histological examinations for the small intestine from the mice in different groups revealed a significant difference (**Fig. 8**). Compared to the small intestines from healthy mice, the small intestines from PBS and Va groups showed massive intestinal epithelial cell shedding and mucosal villi disintegration. The mucosa of the duodenum and jejunum were severely damaged. The comparable injury was not observed in the multivalent vaccine immunized mice, and the small intestine remained intact. There was no difference in intestinal morphology between Vm and normal groups.

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Fig. 7. Representative photographs of the intact small intestine morphology of immunized animals after enterotoxigenic *Escherichia coli* challenge. (A) Small intestine from untreated mice (normal mice); (B, C) Severe diarrhea symptoms of the small intestine were observed in mice of Va and PBS groups; (D, E, F) Small intestine of mice vaccinated with fimbriae, SLS and multivalent antigens.
SLS, STa, LTB and STb.

DISCUSSION

ETEC-induced diarrhea in young domestic animals causes significant economic losses in the livestock industry [21]. However, commercially available vaccines cannot fully target the major fimbriae antigens and ST enterotoxins [22]. In this study, the multivalent vaccine, containing F4, F5, F6, F18 and F41 proteins and a fusion enterotoxin (SLS), was able to provide effective protection against ETEC challenge and elicit robust humoral immune and cytokine responses. Thus, the novel vaccine could be considered a good candidate for preventing ETEC outbreaks in farm animals.

The multivalent vaccine has a protective effect against ETEC infection. The fimbriae antibodies can prevent bacteria from adhering to and invading intestinal epithelial cells. However, the antibodies induced by enterotoxins can neutralize toxins and then inhibit the release of liquid. An obvious symptom of ETEC infection is the damage of the intestinal mucosa [23]. The results of the histological analysis showed that ETEC infection destroyed the structure of the mucous layer and intestinal villus in the small intestine, while mice vaccinated with the novel vaccine displayed morphologically normal intestinal mucosa and intestinal epithelial cells. The higher level of sIgA in Vf and Vm groups also proved that the mucosal immune system provided adequate protection against the ETEC damage. The immunization with the multivalent vaccine reduced the fluid accumulation in the intestinal tract, which may alleviate the symptoms of ETEC-associated diarrhea.

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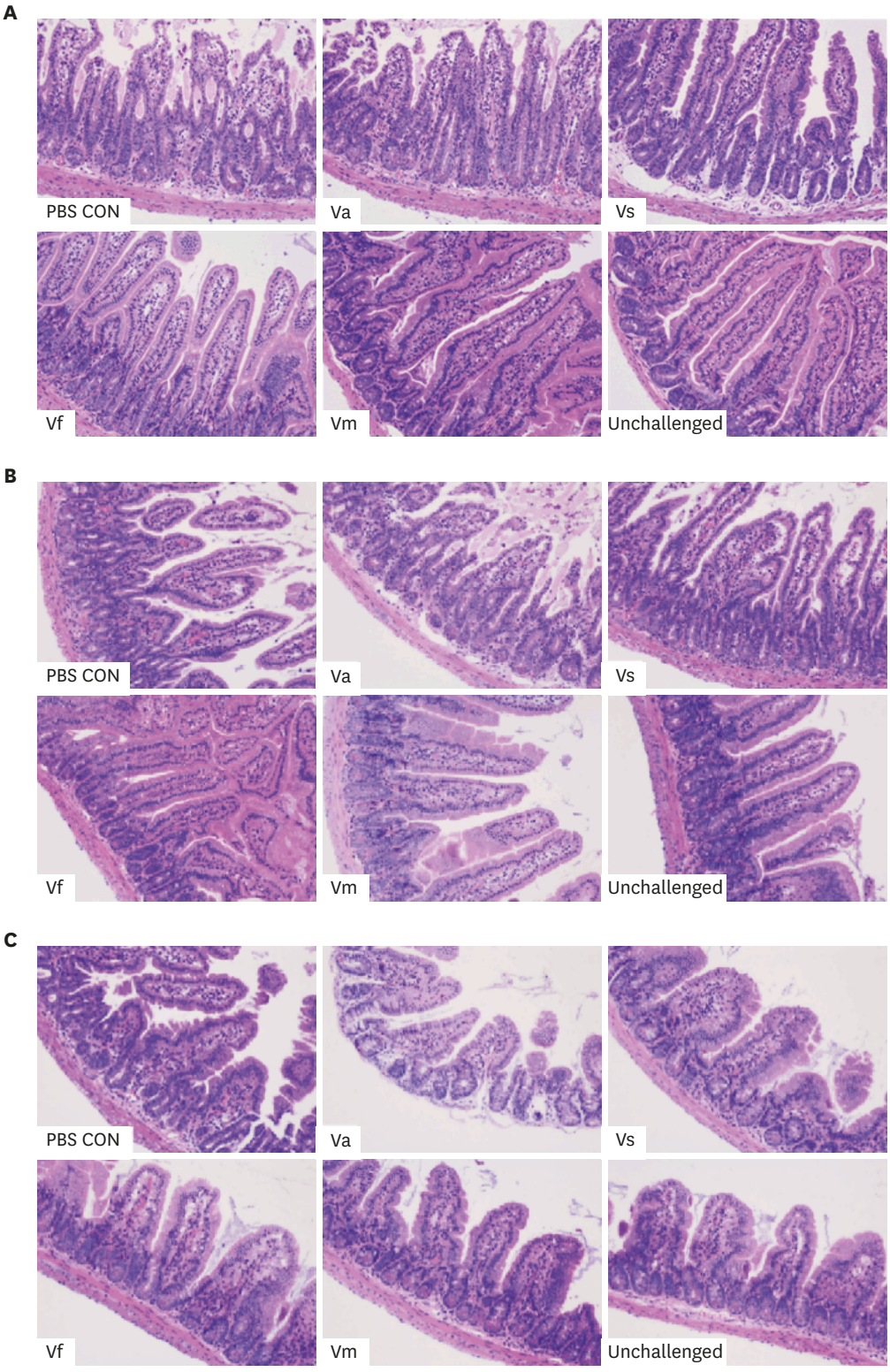

Fig. 8. Histopathology analysis of small intestine lesions from experimental mice after lethal enterotoxigenic *Escherichia coli* challenge (H&E staining, 200×). Histopathological changes were observed in the duodenum (A), jejunum (B) and ileum (C). Histology scores of small intestinal specimens (duodenum (D), jejunum (E) and ileum (F)) of mice. Data are expressed as mean ± SD. Letters above the columns indicate significant differences at the $p < 0.05$ level, as determined by the unpaired *t*-test.
PBS, phosphate-buffered saline.

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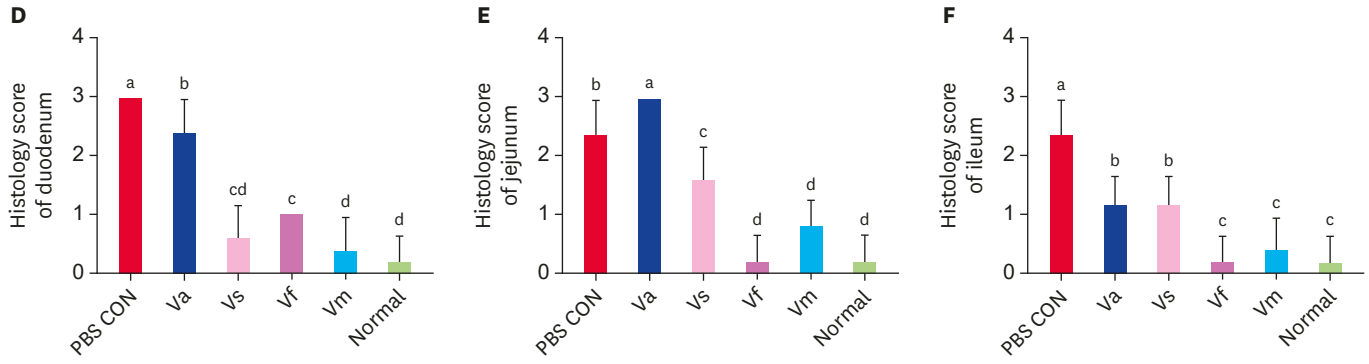

Fig. 8. (Continued) Histopathology analysis of small intestine lesions from experimental mice after lethal enterotoxigenic *Escherichia coli* challenge (H&E staining, 200×). Histopathological changes were observed in the duodenum (A), jejunum (B) and ileum (C). Histology scores of small intestinal specimens (duodenum (D), jejunum (E) and ileum (F)) of mice. Data are expressed as mean ± SD. Letters above the columns indicate significant differences at the $p < 0.05$ level, as determined by the unpaired *t*-test.
PBS, phosphate-buffered saline.

The purified fimbriae proteins induced a high level of specific antibodies against the five targeted antigens. Furthermore, the mice immunized with the fimbriae antigens showed a 70% survival rate after the ETEC challenge, indicating that the vaccine composed of multiple fimbriae antigens could effectively protect animals against ETEC infection. A recent study constructed a multi-epitope fusion antigen with the adhesive subunit of K88, F18, K99, 987P and F41 strains, which exhibited a strong antigenicity in mice, and the anti-fimbriae antibodies inhibited the adhesion of ETEC strains. In addition, these five fimbriae were expressed to intestinal epithelial cell line 1 (IPEC-1) and intestinal epithelial cell line (IPEC-J2) of porcine [24]. These results suggested that specific antibodies generated by vaccination with multiple fimbriae antigens were capable of blocking the adhesion of different ETEC strains to the small intestine, which plays a vital role in preventing ETEC-associated diarrhea.

There are more types of commercial veterinary vaccines containing purified fimbriae antigens than those contain enterotoxin antigens. Colonization factors and enterotoxins are known as the major pathogenic factors of ETEC infection. Thus, it is important to develop effective vaccines containing more fimbriae proteins and enterotoxins. In the present study, the multivalent vaccine composed of both antigens was highly effective against ETEC infection. The survival rate of the Vm group was higher than that of the groups given the vaccines containing only SLS fusion enterotoxin and the fimbriae proteins. LT enterotoxin is considered a powerful adjuvant in parenteral or mucosal immunization, enhancing systemic and mucosal immune responses [25]. Lin et al. [26] showed that piglets vaccinated with purified F4 fimbriae and LT enterotoxin induced robust immune responses, and the protective efficacy of the combination of the two antigens was more effective than immunization with either antigen alone, which is consistent with our results. According to these observations, both fimbriae and enterotoxin are important in protecting against ETEC-associated diarrhea, and the immune responses to fimbriae antigens can be enhanced in the presence of LT enterotoxin. Some veterinary vaccines have optimized formulations that added more purified fimbriae and enterotoxins, suggesting that such strategies are of great research significance.

Maternal vaccination can prevent neonatal diarrhea in the livestock industry, and commercial vaccines are administered parenterally to pregnant sows [27]. For example, Porcilis coli of MSD Animal Health and Suiseng of HIPRA are two representative commercial vaccines for sows, which are given by intramuscular injection. Immunization experiments in the current

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study were performed by parenteral immunity. A previous study observed that adult mice were more sensitive to intraperitoneal injection compared with other routes of challenge, and the results were also found in our pre-test [28]. So, the mice were challenged with ETEC after vaccination by the intraperitoneal route in this study. The significant diarrhea symptoms of the mice indicate that intraperitoneal infection with ETEC is an optional way to evaluate the protective efficacy of novel vaccines.

It is worthwhile considering some deficiencies of the present study. Kunming mice were used to evaluate the immunogenicity of SLS antigen in our previous study, and the mice showed diarrhea symptoms after the ETEC challenge [13]. We choose mice as an animal model to test the immune responses of the novel vaccines for laboratory experiments, and the results showed that the multivalent vaccine could protect mice from ETEC infection. However, some livestock are needed to be included in further research to investigate the effects of the vaccine.

In conclusion, the results of this study demonstrated that the SLS recombinant protein and fimbriae proteins could stimulate robust humoral responses in mice, inducing a high level of specific antibodies against the antigens and eliciting inflammatory cytokines. The multivalent vaccine consisting of the two antigens could effectively protect animals from ETEC infection, maintaining the integrity of the small intestine structure. Furthermore, the vaccine containing both antigens was highly protective than the vaccines containing either antigen alone. These findings indicate that the multivalent vaccine can be a good candidate to prevent ETEC-associated diarrhea in animals.

SUPPLEMENTARY MATERIAL

Supplementary Fig. 1

The expression and purity examination of fimbriae antigens used in the present study. (A) Transmission electron micrographs of five enterotoxigenic *Escherichia coli* strains producing F4, F5, F6, F18 and F41 fimbriae. The bacteria were suspended on a 300 mesh Formvar coated grid and stained with 2% ammonium molybdate. (B) SDS-PAGE analysis of the purified fimbriae proteins. Lane M represents the protein molecular weight marker (labeled in kDa). The protein load was 10 μg/lane.

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