

## Construction of a live attenuated *Salmonella* strain expressing FanC protein to prevent bovine enterotoxigenic *Escherichia coli* and evaluation of its immunogenicity in mice

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**Abstract:** To construct a novel vaccine candidate against bovine enterotoxigenic *Escherichia coli* (ETEC), FanC, the major subunit of K99 fimbriae adhesion, was inserted into secretion plasmid pYA3560 containing a  $\beta$ -lactamase secretion system. This was then transformed into  $\Delta$ asd  $\Delta$ crp *Salmonella* (S.) Typhimurium and designated as JOL950. Secretion of recombinant *fanC* fimbrial antigens was confirmed by immunoblot analysis. Groups of mice were inoculated with single or double doses of JOL950. Another group was used as a negative control. Compared to control mice, all immunized mice had significantly higher levels ( $p < 0.05$ ) of serum immunoglobulin (Ig)G and secretory IgA against FanC. The IgG2a and IgG1 titer assays revealed that immunization highly induced IgG2a compared to that of IgG1, indicating that T helper-1- related cell-mediated immune responses may be elicited by JOL950. The results show that both systemic and mucosal immunities against selected fimbrial antigens of bovine ETEC expressed by a live attenuated *S.* Typhimurium strain are prominently produced in mice immunized with JOL950 via an oral route.

**Keywords:** K99 antigen, *Salmonella* delivery system, bovine enterotoxigenic *Escherichia coli*

### Introduction

Enterotoxigenic *Escherichia coli* (ETEC) pose an economic burden to livestock industries worldwide, causing neonatal diarrhea, the retarded growth, and mortality of the domestic animals [15]. Particularly, bovine ETEC has significantly contributed to the mortality caused by diarrhea in newborn calves [11]. The rise in multi-antimicrobial resistant in ETEC affects the drugs application for the treatment [7]. Instead, vaccination against ETEC as one of the preventive strategies has been developed since the last decade [14]. ETEC virulence relies on the expression of fimbrial colonization factor antigens since bacterial attachment of the host target tissue is instrumental in initiating the infection [16]. Given the pathogenic trait of ETEC, impairment of the adhering mechanism can be an effective defense strategy against ETEC in the early stage of the infection. In newborn calves, the expression of the fimbrial antigen K99 (F5) is responsible for nearly all cases of ETEC infection [11]. Given that the heterogeneous group of ETEC fimbrial adhesins indicates host specificity [11, 16], efficacious protection can be achieved by maternal antibodies against the fimbrial adhesion of a virulent ETEC via colostrum [8, 9] when K99 protein is used as a target antigen for bovine ETEC vaccination.

The expression of eight genes (*fanA* to *fanH*) is required for the biosynthesis of the adhesion K99, which encodes the major subunit protein and other proteins relevant to regulation and transportation [5]. The K99 expression is affected by several endogenous or exogenous factors such as pH, alanine, temperature, the cAMP receptor protein (CRP) [1], the leucine-responsive protein (Lrp), and growth rate [3, 21, 22]. Additionally, in the previous attempts to assess immunogenic potential of K99+ protein against ETEC infection, the purified K99 via an oral route poorly induce secretory immunoglobulin A (sIgA) and serum IgG responses due to gastric proteases [1]. In this context, a bacterial delivery system may elicit the efficient and stable *in vivo* expression of K99 protein in the host by minimizing conformational change of the target protein [4, 12]. The *Salmonella* vectors successfully deliver heterologous antigen to mucosal inductive tissues via an oral route, and then induce immune responses [2, 12]. These findings supported that the use of a live *Salmonella* system delivering K99 antigen via an oral administration can be a robust strategy to eliciting mucosal and systemic immunogenic responses against ETEC infections.

In the present study, we constructed the live attenuated *Salmonella* strain harboring the plasmid pYA3560 carrying *fanC*, a major protein subunit gene of K99 to prevent bovine

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EPEC. This plasmid was designed to translocate the recombinant *fanC* (rFanC) protein fused with  $\beta$ -lactamase signal sequence into the periplasmic space of the *Salmonella* strain. Also, a balanced lethal host-vector mechanism dependent on the bacterial gene aspartate beta-D-semialdehyde dehydrogenase (*asd*) was utilized to retain the plasmid delivering the antigenic protein placed in the *Salmonella* strain [5]. The objectives of the current study are to construct the live attenuated *Salmonella* strain expressing rFanC, and to evaluate immune responses of the strain in a murine model.

## Materials and Methods

### Bacterial strains, plasmids, and growth conditions

All bacterial strains and plasmids utilized in this study are depicted in Table 1. *Escherichia* (*E.*) *coli*  $\chi$ 6212 was used as the host strain for the construction of the Asd<sup>+</sup> plasmids carrying *fanC*. The attenuated *Salmonella* (*S.*) Typhimurium strain,  $\chi$ 8501 constructed by deletion of *crp* and *asd* from wild-type *S.* Typhimurium JOL401 was used for the delivery of antigen protein [12]. The attenuated *Salmonella* strain was used for the delivery of rFanC antigen. The *asd*<sup>+</sup> pYA3560 plasmid was derived from pYA3493 carrying  $\beta$ -lactamase signal sequence-based secretion plasmid and the p15A ori. The *fanC* gene was amplified from the wild-type *E. coli* strain, JOL412 by using the oligonucleotides: FanC\_F:5'-CCGCGAATTCTCTGCGAATACAGTACTA-3' and FanC\_R:5'-CCGCAAGCTTCATATAAGTACTAAGAA-3'. The 546 bp amplified gene fragment, *fanC* was digested with restriction endonucleases, EcoRI and HindIII sites, and cloned into the multiple cloning site of the plasmid pYA3560. The resultant plasmid was designated as the plasmid pYA3560-K99. The plasmid obtained was initially transformed into  $\chi$ 6212 to increase the plasmid stability, and then electrophoretically introduced into  $\Delta$ *asd*  $\Delta$ *crp* *S.* Typhimurium  $\chi$ 8501. The con-

structed strain is designated JOL950. For comparative study, JOL932 constructed by transforming the plasmid pYA3560 into  $\chi$ 8501 was applied as an empty vector control. All strains were incubated in Luria-Bertani (LB) broth or LB agar at 37°C. The *asd* gene deleted bacteria such as *E. coli*  $\chi$ 6212 and *S.* Typhimurium  $\chi$ 8501 were grown in the media supplemented with 50  $\mu$ g/mL of diaminopimelic acid.

### Purification of FanC antigen protein

The PCR product of the *fanC* gene was digested with restriction enzymes EcoRI and HindIII and then was subsequently cloned into the plasmid pET28a. The resultant plasmid was pET28a-K99. The plasmid was transformed into *E. coli* BL21 and was designated JOL794 strain. The recombinant protein was prepared by using an affinity purification process with nickel-nitrilotriacetic acid-agarose support (Qiagen, USA). The purified antigen was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified protein was stored at -70°C until use.

### Preparation of adhesin-specific antisera

To produce anti-rabbit hyperimmune sera against the *fanC* antigen, New Zealand white rabbit was subcutaneously immunized with a mixture containing approximately 250  $\mu$ g of the purified FanC in 1 mL of sterile phosphate buffered saline (PBS) and 1 mL of complete Freund's adjuvant (Sigma-Aldrich, USA). Two booster injections were carried out at week 2 and 4 post-immunization (PI). Blood was sampled at week 2 after the last immunization.

### Western blot analysis

The secretions of the rFanC antigen in JOL950 were validated by immunoblotting. The strains were inoculated in LB broth at 37°C and then were collected at an optical density (OD) of 0.8 at 600 nm (OD<sub>600</sub>). The cell culture centrifuged

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

Strain or plasmid	Relevant characteristics	Source or reference
<i>Escherichia coli</i>		
JOL401	Wild-type F5(K99)+EPEC isolate from bovine	Lab stock
BL21(DE3)pLysS	F <sup>-</sup> , <i>ompT</i> , <i>hsdS<sub>B</sub></i> ( <i>r<sub>B</sub></i> <sup>-</sup> , <i>m<sub>B</sub></i> <sup>-</sup> ), <i>dcm</i> , <i>gal</i> , $\lambda$ (DE3), pLysS, Cm <sup>r</sup>	Lab stock
$\chi$ 6212	F <sup>-</sup> $\lambda$ - $\Phi$ 80 $\Delta$ ( <i>lacZYA-argF</i> ) <i>endA1 recA1 hsdR17 deoR thi-1 glnV44 gyrA96 relA1 <math>\Delta</math>asdA4</i>	Lab stock
JOL794	BL21 containing pET28a-K99	
<i>Salmonella</i> Typhimurium		
$\chi$ 8501	<i>hisG <math>\Delta</math>crp-28 <math>\Delta</math>asdA16</i>	[12]
JOL932	$\chi$ 8501containing pYA3560	This study
JOL950	$\chi$ 8501containing pYA3560-K99	This study
Plasmid		
pET28a	IPTG-inducible expression vector; Km <sup>r</sup>	Lab stock
pET28a-K99	pET28a derivative containing <i>fanC</i> gene	This study
pYA3560	<i>Asd</i> <sup>+</sup> ; p15 ori, $\beta$ -lactamase signal sequence-based periplasmic secretion plasmid	[12]
pYA3560-K99	pYA3560 derivative containing <i>fanC</i> gene	This study

at  $4,000 \times g$  for 20 min and the supernatants were obtained. The supernatants were filtrated by using 0.22- $\mu\text{m}$  pore size Millipore filter followed by precipitation with 20% trichloroacetic acid (TCA). Each sample was loaded and separated using 15% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Millipore, USA). After overnight blocking at 4°C with 5% skim milk in TBS containing 0.1% Tween-20, and the blots were incubated with an adhesin-specific polyclonal antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG (SouthernBiotech, USA). Immunoreactive bands were detected using the WEST-one Western Blot Detection System (iNtRON Biotechnology, Korea) and the multi-wavelength KODAK Image Station 4000<sub>MM</sub> illumination system (Eastman Kodak, USA).

### Preparation of vaccine inoculums

The vaccine candidate JOL950 was cultivated in LB broth at 37°C for 16 h. The cell inoculation diluted 1:20 in fresh LB broth was grown at 37°C to an OD<sub>600</sub> of 0.8. Cell pellets were resuspended to  $1 \times 10^{10}$  colony-forming units per 100  $\mu\text{L}$  of sterile PBS with 0.01% gelatin (BSG). Mice were orally immunized with the prepared strain on the same day.

### Animal experiments

All the animal experiments were conducted with approval from the Chonbuk National University Animal Ethics Committee in accordance with the guidelines of the Korean Council on Animal Care (CBNU2015-00085). Female BALB/c mice ( $n = 30$ ) at 5 weeks of age were randomly assigned into three groups (A to C). The mice in group B were inoculated with a single oral dose of the vaccine strain at 6 weeks of age (*i.e.* week 1 post immunization [PI]), and group C mice were immunized with JOL950 at 6 and 9 weeks of age, respectively (*i.e.* week 1 and 3 PI). The mice in groups A were orally administered with a sterile PBS as a negative control. Mice were deprived of food and water for 4 h prior to immunization until 30 min after the immunization. Blood samples were collected from retro-orbital vein at a two-week interval until week 20 PI. Serum samples were acquired from whole blood via centrifugation at  $4,000 \times g$  for 5 min. Feces were obtained at a two-week interval and suspended with PBS containing 0.1% sodium azide (100 mg/mL). Vaginal secretion specimens were collected in a 50  $\mu\text{L}$  BSG wash. All samples were stored at  $-70^\circ\text{C}$  until the further use.

### Enzyme-linked immunosorbent assay (ELISA)

Titers of FanC antigen-specific IgG, IgA, IgG1, and IgG2a antibody were determined by ELISA in serum as described previously [12]. The level of sIgA against FanC was also assessed in fecal samples and vaginal lavages. Briefly, polystyrene 96-well flat-bottom microtiter plates (Greiner Bio One, Germany) were coated with the purified FanC protein (100 ng/well). The protein was suspended in sodium carbonate-bicarbonate coating buffer (pH 9.6), and was applied with 100  $\mu\text{L}$  volumes in each well. The coated plates were incu-

bated at 37°C for 1 h, followed by an overnight incubation at 4°C. Sera, fecal samples and vaginal secretions obtained from the same experimental group, were diluted 1:600, 1:10 and 1:10, respectively. A 100  $\mu\text{L}$  volume of diluted sample was added to individual wells in duplicate and incubated for 2 h at 37°C. The plates were treated with horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2a, or IgA antibody (SouthernBiotech). Enzymatic reactions were produced by the addition of substrate containing o-phenylenediamine (Sigma-Aldrich). The end titer of the immunoglobulins against FanC was detected by using an automated ELISA spectrophotometer (Tecan, Austria) at the 492 nm. The standard curve was generated by mouse IgG, IgG1, IgG2a, or IgA antibody, and the concentration of each antibody was calculated by using the curve. Results of ELISA are expressed as mean  $\pm$  SD.

### Statistical analysis

The differences between the immunized and non-immunized groups were calculated using non-parametric Mann Whitney rank sum tests. A *p* value less than 0.05 was considered statistically significant.

## Results

### Construction of FanC over-expression strain and purification of rFanC

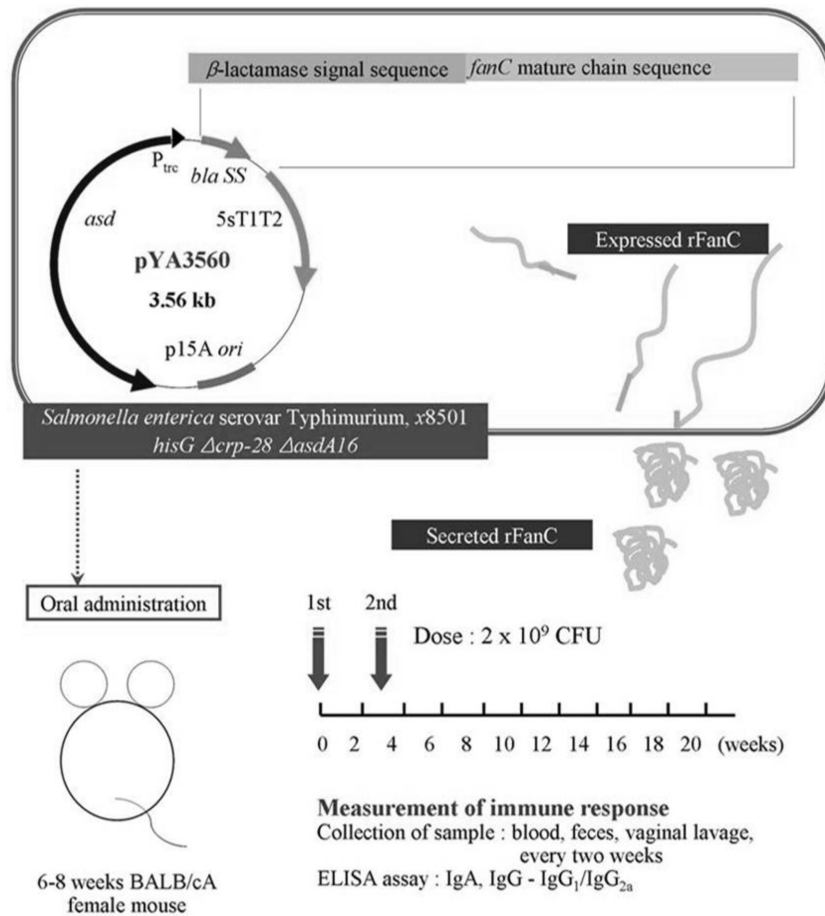
The 546 bp DNA fragment of the *fanC* gene was amplified by the PCR from the DNA template of K99+ ETEC. The PCR product, digested with EcoRI and HindIII enzymes, was inserted into the EcoRI and HindIII sites of pET28a, resulting in pET28a-K99. Insertion of the *fanC* gene onto pET28a vector was determined by restriction enzyme digestion. Increased expression of rFanC was observed by SDS-PAGE. The expected size of the purified antigen was also confirmed via SDS-PAGE. The in-frame fusion of the rFanC was validated by nucleotide sequencing *E. coli* BL21 harboring pET28a-K99 over-expressed rFanC (data not shown).

### Construction of a live attenuated *Salmonella* strain expressing rFanC

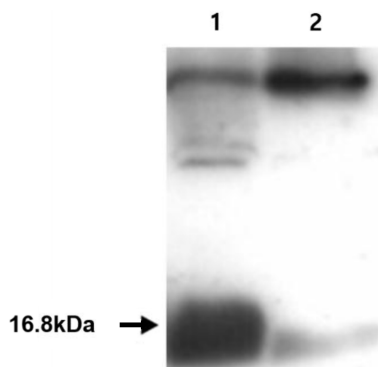
The FanC protein (181 amino acids) was selected as a vaccine candidate antigen against ETEC delivered by a live attenuated *Salmonella* system. The PCR-amplified fragment digested with EcoRI and HindIII enzymes, was cloned into the EcoRI and HindIII sites of pYA3560, resulting in pYA3560-K99 (Fig. 1). Insertion of the *fanC* gene into the pYA3560 vector was confirmed by restriction enzyme digestion. Mutation of the *crp* gene in a  $\Delta\text{asd}$  *S. Typhimurium* strain was determined by the PCR analysis.

### Secretion of recombinant K99 adhesin antigen in JOL950

Western blot analysis was performed by using the TCA-precipitated assay to validate the expression of K99 from



**Fig. 1.** Overview of this study. A live attenuated *Salmonella* Typhimurium strain expressing the recombinant *fanC* (rFanC) antigen, immunization, and measurement of immune responses in mice.

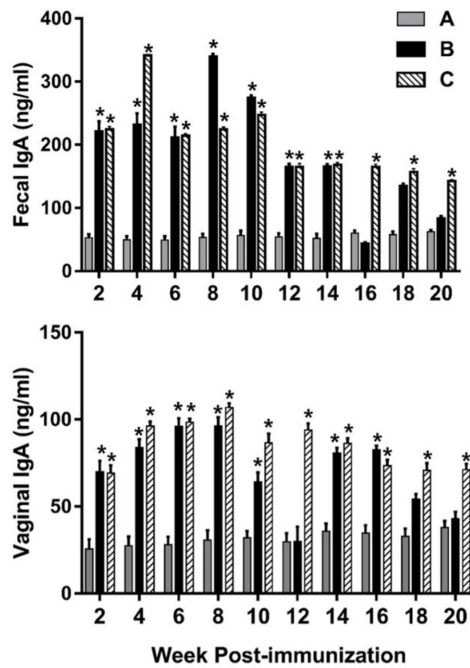


**Fig. 2.** Secretion of rFanC antigen from a live attenuated *Salmonella* strain. FanC expressed by the vaccine was detected by immunoblot using the with rabbit anti-FanC antibodies. JOL932 carrying only pYA3560 was used as a control. The arrow indicates the expected size of the protein. Lane 1, JOL950; Lane 2, vector control.

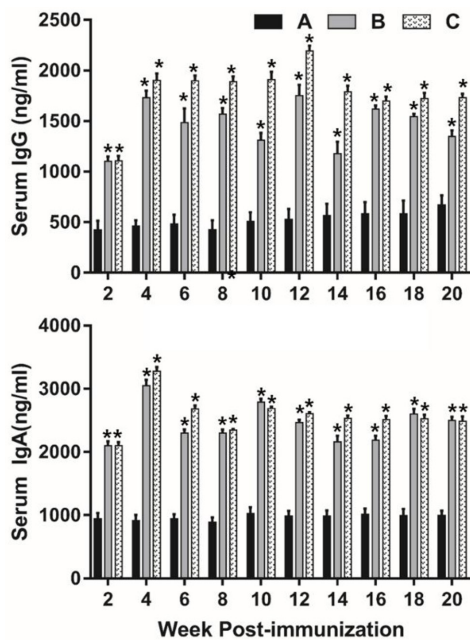
JOL950. JOL932,  $\Delta$ *asd* *S.* Typhimurium harboring the plasmid pYA3560, was utilized as an empty vector control. The expected size (~18.2 kDa) of FanC was observed in the precipitated culture supernatants of JOL950 (Fig. 2).

### Immune responses in immunized mice

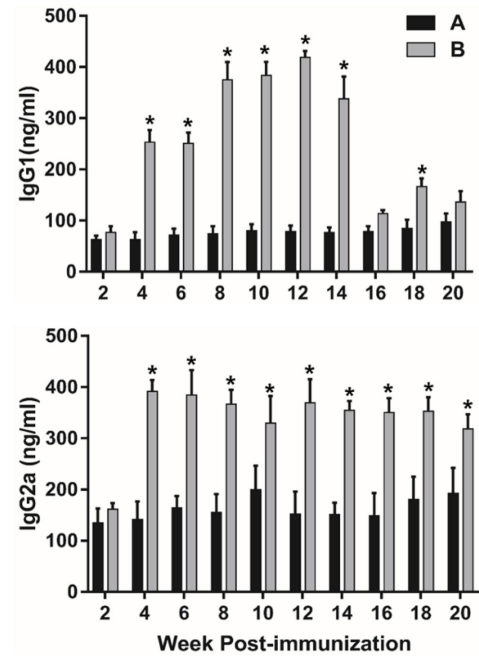
All mice orally administered with the JOL950 survived during the experiment period. The antibody responses against FanC adhesin were measured in the feces and the vaginal secretions from the mice (Fig. 3A and B). The titers of serum IgA and IgG specific to rFanC are presented in Figure 4. At week 2 PI, the level of mucosal sIgA levels detected in both fecal and vaginal lavages increased compared to those in the non-immunized mice. The most increases in mucosal sIgA were observed at week 4 PI, and the elevated levels were continuously detected until week 20 PI. The mice immunized with a double dose of JOL950 showed the enhanced titer of mucosal IgA but did not present significantly increased the level of mucosal IgA compared to those of the mice inoculated with a single dose of the strain ( $p < 0.05$ ). The titers of serum IgG and IgA specific to FanC were also significantly elevated during the observational period compared to those of the negative control ( $p < 0.05$ ) (Fig. 4A and B). The slight increases of IgG in the mice inoculated with a double dose of JOL950 were observed compared to the mice in the group B.



**Fig. 3.** Secretory IgA (sIgA) responses specific to rFanC. The data represent the levels of sIgA antibody against rFanC in feces, and vaginal wash sampled in BALB/c mice orally immunized with  $\chi$ 8501 ( $n = 10$ ). Group A, negative control; Group B, the mice immunized with a single dose of JOL950; Group C, the mice immunized with a double dose of JOL950. \* $p < 0.05$ .



**Fig. 4.** Serum IgG and IgA responses to rFanC. The data represent IgG and IgA antibody levels to rFanC in sera collected from BALB/c mice orally immunized with  $\chi$ 8501 ( $n = 10$ ). Group A, negative control; Group B, the mice immunized with a single dose of JOL950; Group C, the mice immunized with a double dose of JOL950. \* $p < 0.05$ .



**Fig. 5.** Serum IgG2a and IgG1 responses to rFanC. The data represent IgG subclass, IgG2a and IgG1 antibody levels sera collected from BALB/c mice orally immunized with  $\chi$ 8501 ( $n = 10$ ). Group A, negative control; Group B, the mice immunized with a single dose. \* $p < 0.05$ .

#### IgG isotype assessment

Immunogenicity of the K99 adhesin antigens delivered by *S. Typhimurium* were further examined by measuring the level of IgG isotype subclasses IgG2a and IgG1 titers. Both IgG isotype, IgG1, and IgG2a were markedly augmented compared to those of the control group A ( $p < 0.05$ ). The levels of IgG2a specific to FanC were enhanced until the end of the experiment while the titers of IgG1 were decreased at week 16 PI (Fig. 5A and B).

#### Discussion

In the present study, we constructed a vaccine candidate against ETEC using the plasmid pYA3560 expressing FanC delivered by a live attenuated *S. Typhimurium*. The proteins located on the bacterial surface or secreted by the bacterial pathogens are highly immunogenic since they can promptly interact with antigen-presenting cells [13, 18, 22]. Hence, the foreign antigens expressed in the *Salmonella* strain may be translocated into the cell surface or secreted from the cell, and subsequently, tend to elicit the robust immune response in the hosts. Previous studies indicated that the recombinant heterologous antigens expressed to the cell surface membrane of the live-attenuated *Salmonella* vectors were properly delivered and evaluated its immunogenicity to use vaccine candidates [2]. However, the antigen expressed on the surface of delivery vector appeared to be less effective in comparison with the antigen secreted from the delivery vector to

the host environment [2, 4]. The rFanC antigen were fused in frame with the first 35 amino acids of the  $\beta$ -lactamase signal sequence of a secretion vector plasmid pYA3560 [12]. Additionally, the incorporation of the p15A *ori* into pYA3560 improves the survival of *Salmonella* when they harbor rFanC antigen [12]. The immunoblot assays that analyzed the vaccine strains in the culture supernatant demonstrated robust production of the FanC antigens from JOL950 strain. This result supported that the pathogenic *E. coli* fimbrial antigen, FanC was appropriately retained in the constructs and may be effectively secreted from the attenuated *S. Typhimurium* into host animals.

Fimbriae are one of the essential pathogenic factors of ETEC, providing the capacity to attaching and colonizing on the intestinal epithelium of the host [17, 21]. Hence, sIgA specific to fimbrial antigens are instrumental for constituting the intestinal mucosal barriers [6]. The effective mucosal response is induced by directly stimulating Peyer's patches in the gut via the oral administration [9, 20]. The structure and integrity of the target antigen need to be preserved in the oral mucosal vaccine constructs during transit through the gut to elicit the immunogenicity properly [20]. In this regard, a live attenuated *Salmonella* is an ideal delivering vector given its tropism for the mucosal inductive tissues of Peyer's patches [9]. Attenuated *Salmonella* have been applied to express over 50 different heterologous antigens of various origins [10]. In the meantime, mucosal immunity primarily contributes to immune defense system against natural infection by ETEC [2]. In the current study, *S. Typhimurium*  $\chi$ 8501 expressing rFanC remarkably induced the production of IgA in serum, feces and vaginal washes of the orally immunized mice ( $p < 0.05$ ). Additionally, serum levels of IgG against FanC in the immunized mice were significantly elevated compared to those of the control ( $p < 0.05$ ). These results supported that FanC protein secreted in the *S. Typhimurium*  $\chi$ 8501 has immuno-stimulatory property and effectively elicit mucosal and systemic immune responses in the mice.

The IgG subclass distribution is affected by several factors such as secretion of the immunomodulatory cytokine during the process of antigen presentation, expression of co-stimulatory molecules as well as the characteristics of the vaccine antigen [20]. IgG2a is a marker of T helper (Th)-1 cells which direct cell-mediated immune response (CMI) while IgG1 is an indicator of Th-2 cells assisting differentiation of B cells producing antibody [19]. The activation of Th-1 helper cells was predominantly observed following the immunization with a live attenuated *Salmonella* [20, 22]. Our results showed that the level of IgG2a against FanC antigen was observed higher than those of IgG1, indicating that JOL950 may primarily elicit Th-1 type-mediated CMI. Conclusively, our data indicate that both systemic and mucosal immunity can be efficiently induced by the immunization with a double dose of the vaccine constructs. The FanC adhesin protein, the major subunit of K99 ETEC fimbriae antigen can be a promising vaccine candidate against bovine ETEC when

delivered by the live attenuated *Salmonella* system.

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