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Fusion of FedF and SLT-IIe B Subunit is an Candidate Antigen Against Edema Disease of Swine

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Abstract: The DNA sequence encoding the truncated SLT-IIeB and FedF of the *Escherichia coli* Ee strain which is the major immunogenic fragment for the edema disease in piglets were subcloned and fused to the C terminus of Glutathione S-Transferase (GST) in a pGEX-KG expression vector, to obtain a fusion protein expression plasmid pKSF. Fusion protein was expressed as inclusion body. The anti-serum of GST-SF was able to restrain the toxicity of SLT-IIe to Vero-E6 cells and inhibit the adhesion of F18 fimbriae to the porcine small intestinal brush border cells *in vitro*. SPF KM mice were vaccinated subcutaneously at 0 and 2 weeks with 25 μg GST-SF, GST-B and GST-F, respectively and were challenged with 5 times the LD_{50} volume of the Ee strain. The IgG titers against SLT-IIeB or FedF in the GST-SF group were higher and were maintained for a longer time compared to the GST-B and GST-F groups. GST-SF can induce a better immune response and can provide the protection rate of the 70%. These results show the fusion protein GST-SF had stronger immunogenicity and better protective effects against the Ee strain and thus can be used for vaccination against the edema disease of swine.

Key words: Ee strain, SLT-IIeB, FedF, fusion expression, immunogenicity

INTRODUCTION

Toxigenic *Escherichia coli* (*E. coli*), a major pathogen causing diarrhea in humans and animals can be divided into three types, Enterotoxigenic *E. coli* (ETEC), Shiga Toxin-producing *E. coli* (SLTEC, VTEC) and Necrotizing Toxin-producing *E. coli* (NTEC) (Blanco *et al*., 1997; Gannon *et al*., 1993; Kehl *et al*., 1997). SLTEC of which the main serotypes include O138, O139 and O157 is also known as Enterohemorrhagic *E. coli*. SLTEC can produce three isoforms of toxins, SLT-I, SLT-II and SLT-IIe. SLT-I and SLT-II are important virulence factors in human hemorrhagic colitis and hemolytic uremic syndrome and SLT-IIe is an important virulence factor in the Edema Disease (ED) of swine. ED is caused by certain serotypes of SLT-IIe producing *E. coli* and usually occurs in weaned piglets. ED is one of the major diseases threatening the pig farming industry (Karch and Meyer, 1989; Karch *et al*., 1995; Linggood and Thompson, 1987; Ludwig *et al*., 1996). The pathogenesis of ED includes three stages:

- Susceptible pigs are infected by SLTEC
- The F18ab (F107) fimbriae of SLTEC adhere to the porcine small intestinal brush border cells
- The proliferating bacteria secrete SLT-IIe which is absorbed by the intestines and subsequently causes edema and neurological symptoms in infected pigs (Imberechts *et al*., 1992)

F18ab fimbriae and SLT-IIe are two major toxic factors of SLTEC that cause ED (Bertschinger *et al*., 2000; DeGrandis *et al*., 1989).

Conventionally, the generation of ED vaccine is based on the O-antigen of ED-causing *E. coli* but not SLT-IIe or F18ab and thus the efficacy of the vaccine has been low.

SLT-IIe, the immunogenic fragment of ED causing *E. coli*, is a polymer consisting of a 33 ku A subunit and five 7.6 ku B subunits. Antibodies specific for subunit B can prohibit SLT-IIe from binding to its receptors which in turn can inhibit subunit A from entering the cell to produce cytotoxicity. F18ab is composed of five subunits. FedF is the adhesin for F18ab because FedF anti-serum can inhibit the adhesion of $F18^+$*E. coli* to intestinal epithelial cells. In recent years, some researchers have immunized chickens using purified F18ab fimbriae to prepare the egg-yolk antibody against F18ab and found that the antibody could inhibit the adhesion of $F18^+$*E. coli* to intestinal epithelial cells *in vitro* and could have

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protective effects through oral administration (Bertschinger *et al*., 2000). Furthermore, passive immunization with Stx-2e anti-toxin and active immunization with toxoid showed a high vaccination efficacy (Bosworth *et al*., 1996). In the present study, given the anti-adhesion effects of anti-SLT-IIeB and anti-FedF, researchers constructed a chimeric protein consisting of SLT-IIeB and FedF (SF). Researchers further fused the DNA fragment of SF to the C terminus of Glutathione S-Transferase (GST) to obtain a new fusion protein and investigated the biological activity of the fusion protein and the characteristics and immunogenicity of the immune serum.

MATERIALS AND METHODS

Bacterial strain, constructs and positive serum: The ED causing *E. coli* strain Ee (O139, F18$^+$SLTEC) was isolated, verified and stored by the laboratory (Liu *et al*., 2005). The control plasmids pGEX-KG/SLT-IIeB (pKB) (Lin *et al*., 2006) and pGEX-KG/FedF (pKF) were constructed by the laboratory (Liu *et al*., 2008). The pGEX-KG(pK) vector, *E. coli* DH5α competent cells, *E. coli* BL21 (DE3), mouse anti-serum anti-SLT-IIeB and anti-FedF and edema *E. coli* positive serum were all stored in the laboratory. The mouse monoclonal anti-GST antibody was purchased from Sigma (Jie Technology, Chengdu).

Enzymes and reagents: Restriction endonucleases, PCR reagents and T4 DNA ligase were all purchased from TaKaRa (Dalian, China). UNIT-10 Gel Extraction kit was purchased from Shenggong (Shanghai, China). IPTG was purchased from Promega (The original Ping Hao biological, Beijing). Protein marker was purchased from NEB (China Ocean, Beijing) conjugated goat anti-pig IgG antibody was purchased from Huamei (Wuhan, China) protein purification kit was purchased from Amersham Bioscience (Beijing Huamei Bioscience Technology, Ltd. China).

Molecular cloning: Two pairs of primers were designed based on the DNA sequences of SLT-IIe (GenBank No. M21534) and FedF (GenBank No. Z26520) and synthesized by Boao Industry (Shanghai, China). The amplified DNA fragments encode the SLT-IIe B subunit without the signal peptide and transmembrane region (204 bp) and the F18 F subunit without the signal peptide and transmembrane region (897 bp), respectively. The primers P1 and P2 contained cutting sites for BamHI and SacI, respectively. The primers P3 and P4 contained cutting sites for SacI and HindIII. The primer sequences are listed in Table 1. PCR template was prepared as described previously (Liu *et al*., 2005). The 50 μL PCR mixture consisted of 5.0 μL 10×Taq Buffer, 1.0 μL 25 μM $MgCl_2$, 1.0 μL 2 μM dNTPs, 1.0 μL 20 μM 5'-primer, 1.0 μL 20 μM 3'-primer, 1.0 μL Taq DNA polymerase, 30 μL sterile distilled water and 10 μL DNA template. The PCR protocol was as follows: 94°C, 4 min; 94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min, 28 cycles; 72°C, 10 min; 94°C, 5 min; 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, 30 cycles; 72°C, 10 min. The PCR product was analyzed using electrophresis with 0.8% agarose gel and purified using UNIT-10 Gel Extraction kit.

Construction of recombinant expression plasmids and sequencing: The purified PCR products SLT-IIeB (primers: P1 and P2) and FedF (primers: P3 and P4) and the pGEX-KG vector were digested using BamHI/ SacI, SacI/HindIII and BamHI/HindIII, respectively and the digested fragments were purified using electrophresis and gel extraction. The digested products SLT-IIeB, FedF and PK vector were mixed at a ratio of 4:2:1. Following incubation with T4 ligase, the ligation product was transformed into DH5α competent cells. Positive clones were screened and the recombinant constructs were verified using digestion and sequencing analysis.

Expression of fusion protein: Positive bacterial clones were inoculated into LB plates at a dose of 2% and cultured on a shaker till OD 0.4-0.6. IPTG was added at a final concentration of 0.8 μM and the bacteria was cultured for an additional 3 h. Bacteria were collected, mixed with loading buffer and analyzed using SDS-PAGE (12% resolving gel and 5% stacking gel). Western blot analysis was performed as described previously (Guoping *et al*., 2004). Mouse anti-SLT-IIeB anti-serum, mouse anti-FedF anti-serum and mouse anti-GST monoclonal antibody were used as primary antibodies and HRP-conjugated goat anti-mouse IgG was used as a secondary antibody.

Table 1: Primers used to construct the recombinant plasmid pKSF

Genes	Primer	Sequence
SLT-IIeB	P1	5'-CCC GGA TCC TCA GTT AAA CTT CAC C-3'
	P2	5'-CCC GAG CTC GCG GAT TGT GCT AAA GG-3'
FedF	P3	5'-TTT GAG CTC ACT CTA CAA GTA GAC-3'
	P4	5'- TTA AGC TTT GGT CTA CTT ATT ACG CGATG-3'

Preparation of fusion protein anti-serums: Collected bacteria were centrifuged at 8000 g at 4°C for 5 min and the supernatants were discarded. The pellets were resuspended using buffer A (1/10 volume), sonicated and centrifuged at 8000 g at 4°C for 5 min with supernatants discarded. The pellets were washed twice with PBS and resuspended in a mixed solution consisting of 19.7 mL buffer A and 0.3 mL 20% SKL solution. After 30 min to 2 h, the suspension was centrifuged at 12000 g at 4°C for 10 min. The supernatants were transferred and mixed with 20% PEG4000 of which the final concentration would be 0.2%. The 50 mmol L^{-1} oxidized glutathione was added to a final concentration of 1 and 100 mmol L^{-1} reduced glutathione was added to a final concentration of 2 mmol L^{-1}. After 30 min to 2 h, dialysis was performed with PBS for 3 days. Following renaturation, proteins were purified using GST-4B column, measured with concentration and stored at -80°C for use. The 1 mL purified GST-SF (450 μg mL^{-1}) was mixed with 1 mL FIA and inoculated into 4 weeks old rabbits through multi-point injection. After 2 weeks, the rabbits were re-immunized with GST-SF at a concentration of 890 μg mL^{-1}. After 5 weeks, the rabbits were immunized with GST-SF at a concentration of 740 μg mL^{-1} (without FIA) through a slow intravenous injection from the ear. After 1 week, blood was collected from the ear vein and the serum was isolated. The serum antibody titer (antigen: GST-SF) was measured using an agar diffusion test. At a titer of 1:32, the rabbits were killed to acquire the serum. The serum was sterilized using a 22 μm filter, aliquoted and stored at -80°C for use.

***In vitro* activity test of anti-serum**

Neutralization of SLT-IIe cytotoxicity by anti-serum: SLT-IIe was collected (Liu *et al*., 2005) and measured with the CD_{50} for Vero-E5 cells (Sambrook *et al*., 1989; Boyd *et al*., 1993). The expression product of the anti-serum was 2 fold diluted with DMEM in a 96 well cell plate to 50 μL per well. Equal volume Ee culture supernatant (bacteria quantity: $40\times CD_{50}$) was diluted in DMEM. Non-toxin-producing DH5α culture was used as a control. The plates were incubated at 37°C in a 5% CO_2 atmosphere for 72 h and the cell growth was observed under an inverted microscope.

Adhesion test and anti-serum adhesion inhibition test: Brush border cells were prepared (Schmitt *et al*., 1991; Sellwood *et al*., 1975) to a final concentration of 10^6 mL^{-1}. Bacterial suspension was prepared (Parry and Porter, 1978) to a final concentration of 10^9 cfu mL^{-1}. The 50 μL brush border cells and bacteria were added into a small capped bottle. Following slow inversion, the bottle was incubated at 37°C for 30 min. After washing with PBS and centrifugation, cells were stained with Rui-Giemsa dye on a slide for 10 min and observed under a microscope.

Immunogenicity of GST-SF: The 70, 6 weeks old female SPF mice were randomly divided into 5 groups with 14 mice per group including the GST-SLT-FedF (GST-SF) group the GST-SLT-IIeB (GST-B) group, the GST-FedF group, the GST group and the saline control group. Among the 14 mice, 4 were used for measurement of the antibody concentration and the other 10 were used for the immunity protection test. The animals were immunized through subcutaneous injection at a dose of 25 μg/0.2 mL/animal. From 1 week after the initial immunization, blood was collected every other week and the serum was isolated. The sera in one group were mixed together in equal volume and the average antibody concentration of the group was determined using indirect ELISA.

The 2 weeks after the initial immunization, all animals were re-immunized at the same dose. After 2 weeks, the LD_{50} of the Ee strain was measured as described previously (Liu *et al*., 2008). The mice were inoculated with the Ee strain through intraperitoneal injection at a dose of $5\times LD_{50}$ (1.3×10^9CFU). During the following 15 days, the clinical manifestations of the mice were monitored.

RESULTS

Construction of pKSF and sequencing analysis: The recombinant construct was verified by PCR using P1 and P4 as primers and as expected, a 1200 bp band was obtained. Further sequencing analysis revealed that the DNA sequence of the chimera showed a 100% homology to the sequences of SLT-IIeB and FedF in the Ee strain. The recombinant plasmid contained an intact SLT-IIeB fragment and an intact FedF fragment and thus was correctly constructed. Researchers designated this plasmid as pKSF.

Expression and verification of GST-SF: SDS-PAGE analysis of the pKSF expressing *E. coli* BL21 revealed a 63 kDa band which indicated the fusion protein of SLT-FedF and GST (GST-SF). The expression of GST-SF reached the peak at 4 h after IPTG induction. In contrast, the control BL21 which expressed pK only showed a 27 kDa band which indicated the expression of GST. Western blot analysis using purified proteins indicated that GST-SF showed a cross reaction specifically with the mouse anti-FedF antiserum, mouse anti-SLT-IIeB antiserum and mouse monoclonal anti-GST antibody (Fig. 1).

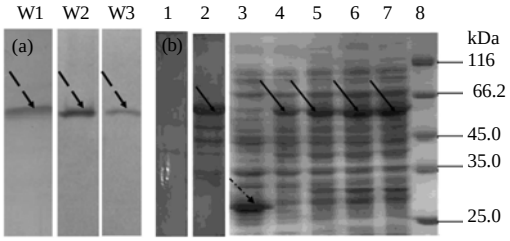

Fig. 1: SDS-PAGE and Western blot analysis of pKSF. a) Western blot analysis of pKSF. Lane W1-W3, desired GST-SF reaction with mouse anti-SLT-IIeB polyclonal antibody, mouse anti-FedF polyclonal antibody, mouse anti-GST monoclonal antibody, respectively; b) SDS-PAGE analysis of pKSF. The arrowheads indicate the band of desired GST-SF (63 kDa) and GST control (27 kDa). Lane 1: supernatant of *E. coli* expressing pKSF; Lane 2: inclusion body of *E. coli* expressing pKSF; Lane 3: pellet of *E. coli* expressing pK; Lane 4-7: pellet of pKSF expressing *E. coli* at 1-4 h after IPTG induction; Lane 8: the molecular mass standards (kDa)

In vitro anti-serum activity test of GST-SF

Neutralization of SLT-IIe cytotoxicity by anti-serum: The results indicated that at a dilution of 1:256, the antiserum could fully neutralize 40×CD_{50} SLT-IIe and subsequently inhibit the cytotoxicity of SLT-IIe to Vero-E6 cells (Fig. 2a). In contrast, the control cells without anti-serum treatment exhibited degeneration, loss, death and dissolution in response to SLT-IIe (Fig. 2b). The second control, the supernatants of the non-toxin-producing DH5α culture, did not show cytotoxicity to Vero-6E cells (Fig. 2c). The third control, normal cells, is shown in Fig. 2d.

Adhesion test and anti-serum adhesion inhibition test: Brush border cells and Ee bacteria were mixed for interaction and then stained with Rui-Giemsa dye and observed under a microscope. The results showed that the bacteria tightly adhered to the brush border cells. At an anti-serum dilution of 1:40, the bacteria did not adhere indicating that the rabbit anti-GST-SF anti-serum could significantly inhibit the adhesion of Ee bacteria to the brush border cells (Fig. 3).

Immunogenicity of GST-SF in mice

Changes of antibody level in mice: The results indicated that mice in the GST-SF group, the GST-F group and the GST-B group showed the highest antibody level at 5, 5 and 4 weeks, respectively after the initial immunization and the antibody level of GST-SF was significantly higher

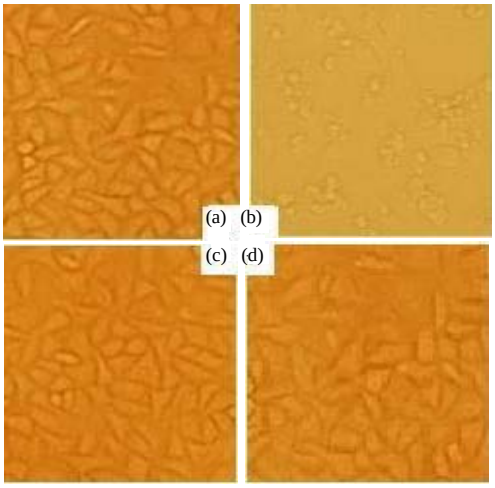

Fig. 2: Toxicity of SLT-IIe to Vero cells and the ability of the antiserum to restrain the toxicity of SLT-IIe. a) The toxicity of SLT-IIe to Vero cells was 100% restrained by antiserum; b) Vero cells attacked by the toxicity of SLT-IIe from the supernatant of Ee culture; c) the supernatant of the DH5α culture was not toxic and d) normal Vero cells as control

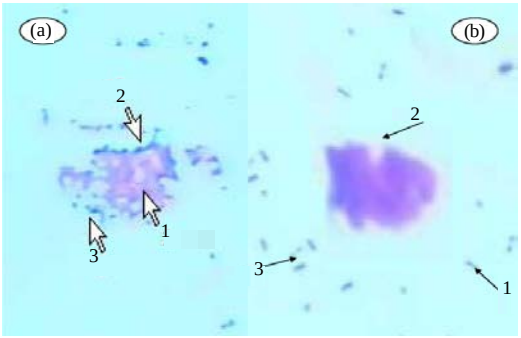

Fig. 3: a) Adhesion of F18-positive *E .coli* to brush border cells from a piglet; b) the anti-adhesion capability of the antiserum. (a) 1: Brush border cell; 2 and 3: bacteria adhered to the cell. (b) 1 and 3: un-adhered bacteria; 2: brush border cell

than that of GST-F and GST-B ($p<0.05$). Furthermore, ELISA results indicated that the GST control and saline control groups showed negative results to SLT-IIeB and FefF (Fig. 4 and 5).

Immune protection test: The results indicated that mice in the GST control and the saline control groups all died within 12 h with bloody foam flowing out from the mice. The necropsy results showed pulmonary bleeding, hepatosplenomegaly and bleeding and flatulence. Toxin-producing bacteria could be detected in

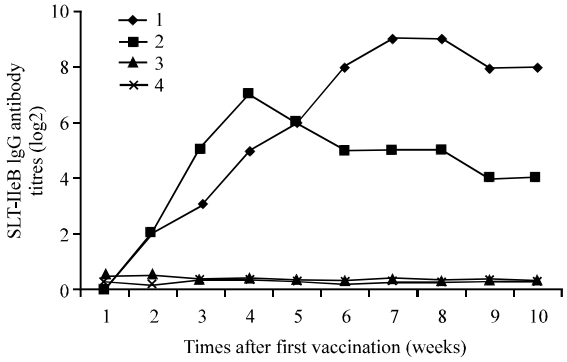

Fig. 4: IgG antibody titers against SLT-IIe or FedF by ELISA at different time of post-vaccination in mice. 1: Immulization by GST-SF; 2: Immulization by GST-F; 3: Immulization by GST; 4: Immulization by Isotonic Na

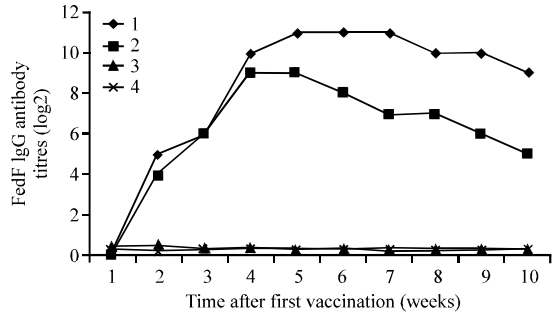

Fig. 5: Antibody titers against FedF at different post-vaccination time points determined by ELISA. 1: Immulization by GST-SF; 2: Immulization by GST-F; 3: Immulization by GST; 4: Immulization by Isotonic Na

all internal organs of the dead mice. For the GST-SF group, 3 out of 10 mice undergoing the immunity protection test died within 6 h and the other 7 showed symptoms of depression and anorexia and liked to get together within the initial 24 h and gradually became normal indicating the protection rate was 70% (7/10). For the GST-F group, 8 mice died within 24 h indicating a protection rate of 20% (2/10). For the GST-B group, 1 mouse died within 6 h and another 5 mice died within 12 h. The other 4 mice showed symptoms of depression and anorexia and liked to get together within the initial 24 h and gradually became normal indicating a protection rate of 40% (4/10).

DISCUSSION

The edema-causing *E. coli* Ee strain was isolated locally by the laboratory. Animal pathogenesis tests showed that the Ee bacteria have a strong virulence in mice. The sterile filtrate of the Ee broth culture was lethal in mice which showed edema by necropsy and caused Vero cell death (Liu *et al*., 2005). Furthermore, intravenous injection into weaned pigs can induce the typical symptoms of edema (Liu *et al*., 2005). Previously, the *FedF*, *SLT-IIeB* and *SLT-IIeA* genes of Ee bacteria have been successfully cloned and extensively studied (Liu *et al*., 2005, 2008; Lin *et al*., 2006; Guoping *et al*., 2004). Based on this research, researchers expressed the fusion protein of SLT-IIeB and FedF in *E. coli*.

With the mechanism study of ED in swine, it has been suggested that to prevent ED occurrence, the ideal vaccine should not only be able to prevent the adhesion of *E. coli* in piglets but also protect the piglets from SLT-IIe secreted by *E. coli*. However, inactivated vaccine based on the O-antigen has been used in China and showed poor efficacy. Hence, to address this issue and given the anti-adhesion effects of both SLT-IIe and FedF, the fusion protein SF might be useful for the development of a new vaccine.

Not all fusion proteins can function or show biological activity as expected, largely because the association of the two proteins can baffle the, conformation changes of each other or the non-neutralizing epitope may overwhelm the neutralizing epitope (Garrity *et al*., 1997). It is therefore necessary to verify the biological activity of fusion proteins. In the present study, researchers first analyzed the antigenic sites of the SF fusion protein using the HLA Epitope binding prediction program (HLA Ligand/Motif DATABASE website). The results indicated that the SF fusion protein showed two epitopes at 35-70 and 96-140 regions which were consistent with the confirmed epitopes in SLT-IIeB and FedF, respectively (Sellwood *et al*., 1975; Garrity *et al*., 1997). Western blot analysis of SF with mouse anti-SLT-IIeB antiserum, mouse anti-FedF antiserum and mouse monoclonal anti-GST antibody all revealed specific bands indicating that the GST-SF fusion protein had good reactogenicity and thus might have the neutralizing epitopes of SLT-IIeB and FedF. Furthermore, the GST-SF antiserum also inhibited the cytotoxicity of SLT-IIe to Vero cells and the adhesion of Ee bacteria to intestinal brush border cells. All these findings indicated that GST-SF had the neutralizing epitopes of SLT-IIeB and FedF and thus can be a candidate antigen for development of a vaccine for ED in swine.

Researchers further inoculated SPF mice with GST-SF, GST-F and GST-B, respectively. The antibody titer measurement results indicated that the GST-SF showed a significantly higher value of average antibody

titer which was also maintained for a longer time, compared to GST-F and GST-B. The results of the immune protection test indicated that GST-SF can better protect cells from attack by toxin-producing Ee bacteria compared to GST-F and GST-B. This observation indicated that the fusion protein had an enhanced immunogenicity compared to isolated GST-F and GST-B. The prediction of the secondary structure of GST-SF using SOPMA software revealed that GST-SF has a 50.57% random coil structure within which some neutralizing epitopes of SLT-IIeB and FedF are located (Parry and Porter, 1978; Smeds *et al*., 2003; Ling *et al*., 2000; Garrity *et al*., 1997). Although, it is possible that the enhanced immunogenicity of the SF fusion protein resulted from the synergy of the two fused proteins, one alternative explanation is that these neutralizing epitopes were fully exposed to increase the neutralizing epitopes. Future research is required to identify these two possibilities.

CONCLUSION

Researchers for the first time, constructed a fusion protein comprising the two major antigens of SLTEC and investigated the biological activity of the fusion protein. Given the good immunogenicity of the recombinant protein and the neutralization capability of its anti-serum, SF can be a good candidate antigen for the development of a vaccine for ED in swine. The study also provides new strategy for the expression of multiple genes and the development of new *E. coli* vaccines.

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REFERENCES

Bertschinger, H.U., V. Nief and H. Tschape, 2000. Active oral immunization of suckling piglets to prevent colonization after weaning by enterotoxigenic *Escherichia coli* with fimbriae F18. Vet. Microbiol., 71: 255-267.

Blanco, J.E., M. Blanco, A. Mora and J. Blanco, 1997. Production of toxin (enterotoxins, verotoxins and necrotoxins) and colicins by *Escherichia coli* strains isolated from septicemic and healthy chickens relationship with viropathogenicity. J. Clin. Microbiol., 35: 2953-2957.

Bosworth, B.T., J.E. Samuel, H.W. Moon, A.D. O'Brien, V.M. Gordon and S.C. Whipp, 1996. Vaccination with genetically modified Shiga-like toxin IIe prevents edema disease in swine. Infect. Immun., 64: 55-60.

Boyd, B., G. Tyrrell, M. Maloney, C. Gyles, J. Brunton and C. Lingwood, 1993. Alteration of the glycolipod binding specificity of the pig edema toxin from globotetraosyl to globotriaosyl ceramile alters *in vivo* tissue targeting and results in a verotoxin-like disease in pigs. J. Exp. Med., 177: 1745-1753.

DeGrandis, S., H. Law, J. Brunton, C. Gyles and C.A. Lingwood, 1989. Globotetraosylceramide is recognized by the pig edema disease toxin. J. Biol. Chem., 264: 12520-12525.

Gannon, V.P.J., M. Rashed, R.K. King and E.J.G. Thomas, 1993. Detection and characterization of the eae gene of shiga-like toxin-producing *Escherichia coli* using polymerase chain reaction. J. Clin. Microbiol., 31: 1268-1274.

Garrity, R.R., G. Rimmelzwaan, A. Minassian, W.P. Tsai and G. Lin *et al*., 1997. Refocusing neutralizing antibody response by targeted dampening of an immunodominant epitope. J. Immunol., 159: 279-289.

Guoping Liu, B.W., M. Liu, Q. He, X. Tang and H. Chen, 2004. Cloning indification and sequence analysis of the a subunit gene of SLT-?e toxin. Progress Vet. Med., 25: 122-125.

Imberechts, H., H. De Greve and P. Lintermans, 1992. The pathogenesis of edema disease in pigs: A review. Vet. Microbiol., 31: 221-233.

Karch, H. and T. Meyer, 1989. Single primer pair for amplifying segments of distinct Shiga-like-toxin genes by polymerase chain reaction. J. Clin. Microbiol., 27: 27512-27571.

Karch, H., H. Russmann, H. Schmidt, A. Schwarzkopf and J. Heesemann, 1995. Long-term shedding and clonal turnover of enterohemorrhagic *Escherichia coli* O157 in diarrheal diseases. J. Clin. Microbiol., 33: 1602-1605.

Kehl, K.S., P. Haven, C.E. Behnke and D.W. Acheson, 1997. Evaluation of the premier EHEC assay for detection of Shiga toxin 2 producing *Escherichia coli*. J. Clin. Microbiol., 35: 2051-2054.

Lin, Y., G. Liu and B. Wu, 2006. Cloning, sequence analysis and expression of b subunit of Shiga-like toxin IIe gene in *Escherichia coli*. J. Huazhong Agric. Univ., 25: 474-478.

Ling, H., N.S. Pannu, A. Boodhoo, G.D. Armstrong, C.G. Clark, J.L. Brunton, R.J. Read, 2000. A mutant Shiga-like toxin IIe bound to its receptor Gb_3: Structure of a group II Shiga-like toxin with altered binding specificity. Structure, 8: 253-264.

Linggood, M.A. and J.M. Thompson, 1987. Verotoxin production among porcine strains of *Escherichia coli* and its association with oedema disease. J. Med. Microbiol., 24: 359-362.

Liu, G., A. Guo and B. Wu, 2008. Cloning,expression and characterization of the F subunit gene of F18 fimbriae of the Ee strain. Chinese J. Vet. Sci., 28: 479-484.

Liu, G., B. Wang, M. Liu, Q. He, X. Tang and H. Chen, 2005. Detection, identification and biological characterization of *Escherichia coli* eiciting edema of piglet. Chin. J. Vet. Sci., 25: 31-33.

Ludwig, K., M. Bitzan, S. Zimmermann, M. Kloth, H. Ruder and D.E. Muller-Wiefel, 1996. Immune response to non-O157 Vero toxin-producing *Escherichia coli* in patients with hemolytic uremic syndrome. J. Infect. Dis., 174: 1028-1039.

Parry, S.H. and P. Porter, 1978. Immunological aspects of cell membrane adhesion demonstrated by porcine enteropathogenic *Escherichia coli*. Immunology, 34: 41-49.

Sambrook, J., E.F. Fritsch and T. Maniatis, 1989. Molecular Cloning: A Laboratory Manual. 2nd Edn., Cold Spring Harbor Laboratory Press, New York, USA., ISBN-13: 9780879693091.

Schmitt, C.K., M.L. McKee and A.D. O'Brien, 1991. Two copies of Shiga-like toxin II-related genes common in enterohemorrhagic *Escherichia coli* strains are responsible for the antigenic heterogeneity of the O157:H-strainE32511. Infect. Immun., 59: 1065-1073.

Sellwood, R., R.A. Gibbons, G.W. Jones and J.M. Rutter, 1975. Adhesion of enteropathogenic *Escherichia coli* to pig intestinal brush borders: The existence of two pig phenotypes. J. Med. Microbiol., 8: 405-411.

Smeds, A., M. Pertovaara, T. Timonen, T. Pohjanvirta, S. Pelkonen and A. Palva, 2003. Mapping the binding domain of the F18 fimbrial adhesin. Infect. Immunity, 71: 2163-2172.