Title: Strategies to include STa antigen in vaccine development against porcine post-weaning diarrhea (PWD); **NPB #08-005**

Investigator: Weiping Zhang

Institution: South Dakota State University

Date Submitted: Mar. 2nd. 2010

Summary
Enterotoxigenic *Escherichia coli* (ETEC) strains are a major cause of diarrhea disease in humans and farm animals. *E. coli* fimbriae or colonization factor antigens (CFAs) and enterotoxins including heat-labile (LT) and heat-stable (ST) are the key virulence factors in ETEC diarrhea. Unlike fimbriae or LT, STa has not been much included as an antigen in vaccine development against ETEC diarrhea because of its poor immunogenicity. STa becomes immunogenic only after being coupled with a strongly immunogenic carrier protein. However, native or shorter STa antigens either had to retain toxic activity in order to become antigenic or elicited anti-STa antibodies that were not sufficiently protective. In this study, we genetically mutated porcine LT (pLT) gene for a $pLT_{192(R\rightarrow G)}$ toxoid and STa (pSTa) gene for three full-length pSTa toxoids [$STa_{11(N\rightarrow K)}$, $STa_{12(P\rightarrow F)}$, and $STa_{13(A\rightarrow Q)}$], and used the full-length pLT_{192} as an adjuvant to carry the pSTa toxoid for 'pLT_{192}:pSTa-toxoid' fusion antigens. Rabbits immunized with 'pLT_{192}:$pSTa_{12}$' or 'pLT_{192}:$pSTa_{13}$' fusion protein developed high titers of anti-LT and anti-STa antibodies. Furthermore, rabbit antiserum and antifecal antibodies were able to neutralize purified cholera toxin (CT) and STa toxin. In addition, preliminary data suggested that suckling piglets born from a sow immunized with the 'pLT_{192}:$pSTa_{13}$' fusion antigen were protected when challenged with a STa-positive ETEC. This study demonstrated that pSTa toxoids are antigenic when fused with a pLT toxoid, and elicited anti-LT and anti-STa antibodies were protective. This fusion strategy could provide instructive information to develop effective toxoid vaccines against ETEC associated diarrhea in animals and humans.

Introduction: An overview of the researchable question and its importance to producers.
Enterotoxigenic *Escherichia coli* (ETEC) strains that colonize host small intestines, invade epithelial cells and produce one or more enterotoxins are a major cause of diarrheal disease in humans and farm animals. The virulence determinants of ETEC in diarrhea include fimbrial adhesins and enterotoxins (Alexander, 1994; Francis, 2002; Moon, 1990; Moon and Burn, 1993; Smith and Linggood, 1971; Wilson and Francis, 1986; Berberov et al., 2004; Zhang et al., 2006). Fimbrial adhesins mediate attachment between bacteria and host epithelium cells and facilitate colonization. Enterotoxins including heat-stable (STa and STb) and heat-labile (LT) enterotoxins (Hirayama, 1995; Hol et al., 1995; O'Brien and Holmes, 1996; Sear and Kaper, 1996) disrupt intestinal fluid homeostasis and cause fluid and electrolyte hyper-secretion through activation of adenyl cyclase (by LT) or guanylate cyclase (by STa) in small intestinal epithelial cells (Hughs et al., 1978; Moon, 1978). ETEC strains isolated from young pigs with diarrhea express LT , STa , STb, Stx2e, EAST 1(enteroaggregative *E. coli* heat stable toxin type 1) alone or combined (Francis, 2002; Frydendahl, 2002; Zhang et al., 2007). Recent experimental studies indicated that porcine ETEC strains expressing LT, STb, or STa alone are sufficiently virulent to cause diarrhea in young pigs (Berberov et al., 2004; Zhang et al., 2006; 2008)

These research results were submitted in fulfillment of checkoff-funded research projects. This report is published directly as submitted by the project's principal investigator. This report has not been peer-reviewed.

For more information contact:
National Pork Board • PO Box 9114 • Des Moines, IA 50306 USA • 800-456-7675 • Fax: 515-223-2646 • pork.org

.

Porcine ETEC-associated diarrhea, especially post-weaning diarrhea (PWD), causes a substantial economic loss to the swine producers (Svensmark et al., 1989; Harvey et al., 2005). Currently, there are no effective vaccines available to protect weaned pigs against ETEC infection. Experimental vaccines developed from fimbrial antigens alone, particularly he K88 fimbria, showed only limited protection against ETEC infection (Van den Broeck et al., 1999). In addition, various ETEC fimbriae are antigenically different, thus, vaccines developed from one specific fimbria could not provide protection against an ETEC strain expressing a different fimbria (Van den Broeck et al., 1999; Walker, 2005). Moreover, recent evidence suggests that fimbriae may not function as protective antigens in the setting of naturally acquired infections and re-infections (Boedeker, 2005). Consequently, enterotoxin antigens have been re-emphasized recently (Walker, 2005). Antitoxin vaccines currently under development largely use LT as the antigens because of its strong immunogenicity and antigenicity. The ST antigens cannot be used directly as a vaccine component because they are poorly immunogenic unless coupled to a carrier protein or present as a fusion protein. Although a recent study suggested that anti-LT immunity may provide a broader protection (Frech et al, 2008), experimental vaccines that induce anti-LT immunity alone provide protection only against infection from LT-producing ETEC strains, they cannot extend protection against ETEC strains producing STa toxin (Frantz and Robertson, 1981; Frantz and Mellencamp, 1983). As over two thirds of the human ETEC strains and more than one quarter of the porcine ETEC strains are STa positive (Gaastra and Svennerholm, 1996; Nirdnoy et al., 1997; Wolf, 1997, Qadri et al., 2000; Reischl et al., 2004; Shaheen et al., 2004; Girard et al., 2006; Zhang et al., 2007), the STa antigen must be included in developing effective vaccines against ETEC infection.

The porcine STa protein that consists of 18 amino acids (19 amino acids for human STa) is poorly immunogenic. Therefore, to include STa as a vaccine component, we must facilitate the STa immunogenicity. Furthermore, STa is sufficiently virulent to cause diarrhea, thus a native STa cannot be used for developing safe vaccines. It was reported that shorter synthetic STa peptides or STa with disulfide bonds disrupted showed toxicity reduction (Svennerholm et al., 1988; Yamasaki et al., 1988, 1990; Hirayama, 1995; Batisson et al., 2000; Batisson and Vartinian, 2000). Synthetic hSTa peptides had the 12th, 13th, or 14th amino acid residue substituted showed a great reduction in toxicity (Yamasaki et al., 1988; Hirayama, 1995). However, these shorter synthetic or disulfide bond-disrupted STa peptides were either not immunogenic or failed in inducing protective immunity. It was also reported that STa immunogenicity could be enhanced when a shorter hSTa or an hSTa mutant (with disulfide bond-disrupted) was genetically fused to a carrier protein, such as the B subunit of cholera toxin (CT) or hLT (Sanchez et al., 1986; 1988; Sanchez Holmgren, 1988; Clements, 1990). However, anti-STa immunity from these fusion antigens was not sufficiently characterized, and retention of STa toxicity in the fusion proteins caused safety concerns for their application in vaccine development (Clements, 1990; Boedeker, 2005).

No studies have been conducted to enhance pSTa immunogenicity and its potential vaccine application against porcine ETEC infection. In this study, we mutated the porcine *est*A gene at nucleotides corresponding to the 11th, 12th, and 13th amino acids (that are homologous to the 12th, 13th, or 14th amino acid of hSTa) for pSTa toxoids. These STa toxoids had all disulfide bonds retained and their toxicity was tested in vitro (cyclic AMP ELISA) and in vivo (porcine gut loop assay and challenge studies in a piglet model). We then genetically fused the mutated full-length porcine *eltAB* and *estA* genes for 'pLT$_{192}$:pSTa-toxoid' fusion proteins. Purified fusion antigens were used to immunize rabbits to assess anti-LT and anti-STa antigenicity and potential application in vaccine development.

Objectives:

The objectives of this proposed research are to develop strategies to increase immunogenicity of the heat-stable enterotoxin (STa) and to include STa as an antigen for developing effective vaccines against porcine post-weaning diarrhea (PWD). PWD is economically one of the most important diseases in the swine industry. It is estimated that PWD causes near $80 million loss each year in the U.S. swine producers. Yet, there are no effective vaccines commercially available to protect young pigs from PWD. Difficulties in developing effective vaccines against PWD include multiple virulence factors involved in this disease. It becomes clear that only multivalent vaccines consisting of antigens from all virulence determinants of the disease will provide effective protection. Heat-stable type A (STa) is a virulence determinant in PWD, thus, it is a must to include STa antigen as a vaccine component. However, the STa are poorly immunogenic and the native STa are toxic sufficiently to cause diarrhea in young pigs. Therefore, native STa can not be used directly as antigens in vaccine development. This research will use molecular techniques to mutate the STa toxin gene for low- or non-toxic STa antigens, and then genetically fuse the safe STa antigen with a low-toxic LT mutant (LT$_{192}$) for a gene tic fusion protein (LT$_{192}$:mSTa). This genetic fusion protein will enhance the STa immunogenicity and

stimulate host to produce anti-STa antibodies. Since this fusion protein also contains the LT antigens, it can be excellent antigens for developing a multivalent vaccine against PWD.

Materials & Methods:

Bacterial strains and plasmids. A porcine *E. coli* filed isolate G58-2 (Francis and Wilgohs, 1991) was used for constructing experimental strains in this study. Plasmid 987P, kindly provided by Dr. D. Schifferli at University of Pennsylvania (Schifferli and Alrutz, 1994), was used to express 987P fimbria in the G58-2 and STa constructs. Plasmid pACYC184 (Promega, Madison, WI) and TOPO TA cloning vector (Invitrogen, Carlsbad, CA) were used to clone and express recombinant and mutated porcine STa genes, and LT and STa chimeric genes. All constructs were cultured in LB medium supplemented with 20 µg/ml chloramphenicol or 50 µg/ml ampicillin (Table 1).

Porcine *estA* gene cloning and mutation. The porcine STa gene (*est*A) was PCR amplified with genomic DNA from a porcine ETEC field isolate 04-21018 ($F18^{+}STa^{+}STb^{+}Stx2e^{+}$) and designed primers STaSfcI-F and STaEagI-R (Table 2). The forward primer contains a SfcI restriction site and the reserve primer includes an EagI restriction site. PCR was performed at a PTC-100 thermal cycler (BIORAD, Hercules, CA) in 50 µl of reaction containing 1X *Pfu* DNA polymerase buffer (with Mg++), 200 nM dNTP, 0.5 µM of each forward and reverse primers, and one unit of *Pfu* DNA polymerase (Strategene, La Jolla, CA). Amplified products were separated by 1% agarose (FMC Bioproducts, Rockland, MA) gel electrophoresis and purified using a QIAquick Gel Extraction Kit by following the manufacturer's instruction (QIAGEN, Valencia, CA). Purified PCR products and plasmid pACYC184 were digested sequentially with SfcI and EagI restriction enzymes (New England Biolab, Ipswitch, MA). Digested *estA* gene and vector pACYC184 products were purified and then ligated with T4 DNA Ligase (New England BioLab). Two microliters of T4-ligated products were introduced into construct 8227 (G58-2/987P) competent cells by standard electroporation (Ausubel et al., 1999). Positive colonies were screened by PCR initially and then sequenced to ensure that the cloned gene was inserted in the reading frame.

To substitute the 11th, 12th, and the 13th amino acids of the pSTa toxin, we designed specific PCR primers to substitute nucleotides encoding these three residues (Table 2). For a STa_{11} toxoid gene, we used the recombinant STa DNA as a template and amplified the 5' end of the *estA* gene in a PCR using the 184EcoRV-F and the $pSTa_{11K}$-R primer in one PCR reaction, and the 3'end of the *estA* gene in another PCR with the $pSTa_{11K}$-F (complementary to the $pSTa_{11K}$-R primer) and pBREagI-R primer. Then, we overlapped the 5'-end and the 3'-end fragments in a splice overlap extension (SOE) PCR to construct an *estA* gene with mutation at the 11th amino acid residue. Similarly, we mutated the *estA* gene at nucleotides coding the 12th and 13th amino acids with respective primers.

STa competitive ELISA to detect STa protein expression among recombinant and mutant strains. Overnight grown cultures of the STa recombinant and mutant strains, and a negative control strain were used for STa competitive ELISA as described previously (Lockwood and Robertson, 1984; Zhang et al., 2008). Briefly, strains were cultured in LB medium overnight, and grown culture was measured with optical density (OD). Equivalent amount of cells from each strain was used for subculture in 4 AA medium, and 4 AA culture supernatants were used in ELISA. A STa ELISA plate was coated with STa ovalbumin-conjugate (1.25 ng per well), and blocked with 2.5% casein blocking buffer (2.5% casein in 0.3 N NaOH, pH 7.0). Seventy-five microliters of culture supernatant from each strain (in triplicate) and 75µl of anti-STa serum (1:10,000 dilution) were mixed and added to each well, followed by an incubated at 37 ºC for 2 hrs on a shaker (180 rpm). After three washes, plates were blotted to dry, incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (IgG, 1:10,000 dilution) (Sigma, St. Louis, MO) at 37 C for 1 h, and reaction of bound IgG with ABST substrate was measured at 405 nm.

Cyclic GMP ELISA to detect toxicity in STa mutants. Toxicity of the recombinant and mutated STa proteins was tested using a direct cyclic GMP enzyme immunoassay kit (Correlate™ EIA, Assay Designs, MI). Bacterial culture supernatant was tested for stimulation of an increase of intracellular cGMP levels in T-84 cells (ATCC #CCL-248). The cGMP levels were measured in cGMP ELISA by following manufacturer's instruction (acetylated version). Briefly, $1x10^5$ T-84 cells were seeded at each well in a 24-well plate. After removing the Dulbecco's modified Eagle medium (DMEM/F12; GIBCO/Invitrogen, Grand Island, NY), each well was added with 75 µl overnight-grown (in 4AA medium) supernatant from each strain (in duplicate). Cells were lysed with 0.1 M HCl after 2 h incubation. A 100 µl of lyses supernatant was mixed with kit-supplied conjugates and antibody reagents, and the mixture was added to each well of the

supplied EIA plate. After incubation on a shaker at 500 rpm at room temperature for 2 h, plates were washed and blotting dried, and reacted with pNpp substrate solution. The OD was measured at 405 nm after 20 min development.

Detection of STa biological activity in ligated porcine intestinal loops and animal challenge studies. Biological activity of the STa proteins from the recombinant and mutant strains was examined in a porcine ligated loop assay. Two x 10^9 CFUs of overnight-grown crude culture from each strain were injected into each ligated loop. After 8 h post-inoculation, the length of each loop (cm) and amount of fluid accumulated in each loop (gram) were measured. The ratio of fluid accumulation to the loop length (g/cm) was calculated as an index of enterotoxic activity.

Toxicity of each STa mutant strain was also examined in animal challenge studies. A group of four randomly selected 3-day old gnotobiotic piglets were orally inoculated with 3 x 10^9 CFUs overnight-grown culture of each mutant strain, the STa recombinant, or the negative control strain. During the 24 h post-inoculation period, piglets were closely monitored for clinical signs of disease, including vomiting, diarrhea, dehydration, lateral recumbency, and lethargy. All animal studies complied with the Animal Welfare Act, followed the *Guide for the Care and Use of Laboratory Animals (21a)*, and were under the supervision of South Dakota State University's Institutional Animal Care and Use committee.

Construction of 'pLT$_{192}$:pSTa$_{12}$' and 'pLT$_{192}$:pSTa$_{13}$' chimeric genes. A recombinant porcine *eltAB* gene that codes LT toxin and a mutated *eltAB* gene coding LT$_{192}$ strains were cloned in our laboratory (Zhang and Francis, submitted). A 'Gly-Pro-Gly-Pro' (gggccggggccc) linker (Arakawa et al., 2001) was used to connect the mutated *elt*AB and *est*A genes. PCR primers pLT:STa-R and pSTa:LT-F were specifically designed so that they contained nucleotides of the 3' end of the *eltAB* gene (without the stop codon), the linker, and the 5' end of the *estA* gene (without the precursor), respectively. A PCR using primer 184EcoRV-F and pLT:STa-R and plasmid pLT$_{192}$ as the template amplified the mutated *eltAB* gene, the linker and the 5' end of the mutated *estA* gene. A second PCR using pSTa:LT-F and pBREagI-R primers and DNA from the STa mutant plasmid pSTa$_{12}$ generated the fragment covering the 3' end of the mutant *eltAB* gene (no stop codon), the linker and the mutant *estA* gene (no precursor). A SOE PCR connected the mutant *elt*AB and the mutant *est*A genes with the linker for a chimeric gene. This resultant chimeric gene was further amplified with 184EcoRV-F and pBREagI-R primers and then digested with SfcI and EagI enzymes. Digested products were purified and cloned into vector pACYC184 at the SfcI and EagI sites with T4 DNA ligase (Invitrogen). Two microliters of T4 ligated products were introduced into G58/987P host cells with standard electroporation. Positive colonies were screened by PCR and then DNA sequenced to ensure the cloned 'pLT$_{192}$:pSTa$_{12}$' fusion gene was inserted in reading frame.

Similarly, a 'pLT$_{192}$:pSTa$_{13}$' chimeric gene was constructed with pSTa$_{13Q}$-F and pSTa$_{13Q}$-R primers and plasmid pSTa$_{13}$. This 'pLT$_{192}$:pSTa$_{13}$' chimeric gene was also cloned into vector pACYC184 and expressed in a G58/987P host cell. In addition, chimeric genes 'pLT$_{192}$:pSTa$_{12}$' and 'LT$_{192}$:pSTa$_{13}$' were amplified by 184EcoRV-F paired with STa12NS-R and STa13NS-R, and cloned into the TA clone vector pBAD-TOPO and expressed in TOPO 10 cells (Invitrogen), respectively.

Detection of LT and STa in 'pLT$_{192}$:pSTa-toxoid' fusion proteins. The 'pLT$_{192}$:pSTa$_{12}$' and 'pLT$_{192}$:pSTa$_{13}$' constructs were grown in Casamino acids and yeast extract broth with lincomycin (45 µg/ml) and ampicillin (50 µg/ml) at 37 ºC overnight. The overnight-grown culture was centrifuged at 3000 x g for 20 min, and pellets were collected for total protein preparation using bacterial protein extraction reagent (B-PER, in phosphate buffer; Pierce, Rockford, IL).

Thirty microliters of prepared total proteins were used for detecting LT and STa in a standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immuno- blot assay (Ausubel et al., 1992). Transferred membrane blotting was blocked with 2% fat-free milk overnight at 4 ºC and then incubated with anti-CT (Sigma) and anti-STa sera, respectively. After three washes, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (Sigma; 1:5,000 dilution) for 1 h. After a final round of washes, peroxidase bound to the fusion proteins on the membranes were detected with a SuperSignal West Pico Chemiluminescent Substrate kit (Pierce).

Rabbit immunization with purified 'pLT$_{192}$:pSTa$_{12}$' and 'pLT$_{192}$:pSTa$_{13}$' fusion proteins. Fusion proteins 'pLT$_{192}$:pSTa$_{12}$' and 'pLT$_{192}$:pSTa$_{13}$' expressed in the TOPO 10 cells were purified using B-PER (Pierce) and Ni-NTA Agarose (QIAGEN) by following the manufacturer's protocols. Briefly, overnight-grown cultures (in Casamino acid and yeast medium) were pelleted and lysed in B-PER reagent. Total proteins in cell lysis were incubated with Ni-TNA resin, followed by washes and elution. The 6xHis-tagged TA cloned fusion proteins were purified and stored at -20 ºC until use.

A group of two adult rabbits were immunized intramuscularly (IM) with 100 µg purified ‘pLT_{192}:$pSTa_{12}$’ or ‘pLT_{192}:$pSTa_{13}$’ fusion protein in equal volume of Freund’s incomplete adjuvant (Sigma). Two booster injections were followed at biweekly intervals. One rabbit without immunization served as the negative control. Blood and fecal samples were collected before and 14 days after each injection. Collected serum and resuspended fecal samples were stored at -80 ºC until use.

Rabbit serum antibody titration Cholera toxin (CT; Sigma) and STa ovalbumin-conjugate were used as antigens to titrate anti-LT and anti-STa antibodies from rabbit antiserum and anti-fecal samples, respectively. For anti-LT_{192} antibody titration, we coated an ELISA plate with GM1 (400 ng/well) as GM1-ELISA described previously (Zhang et al., 2006). Rabbit antisera (1:50 diluted in PBS; in triplicate) were used as the primary antibodies (in a binary dilution), and HRP-conjugated goat-anti-rabbit IgG as the secondary antibody. To titrate anti-STa antibodies, we coated an ELISA plate with STa ovalbumin-conjugate (1.25 ng/well), and used rabbit anti-serum or anti-fecal antibody samples (1:50 diluted in PBS; in triplicate) as the primary antibodies and HRP-conjugated goat-anti-rabbit IgG or IgA the secondary antibodies. The OD was measured at 405 nm with a plate reader after 20 min of development in peroxidase substrates (KPL). The titration end-point was determined as the reciprocal of the interpolated dilution giving an OD unit above 0.4 after subtraction of background (Lapa et al., 2008; Zhang and Francis, submitted). Antibody titers were expressed as the $\log_{10}$ of the reciprocal dilution.

Anti-LT and anti-STa antibody neutralization Neutralization of CT toxin by rabbit antiserum (anti-LT antibodies) was examined using a cAMP EIA kit (Invitrogen) and T84 cells. For anti-STa antibody neutralization, we used a cGMP EIA kit (Assay Design, Ann Arbor, MI) and T84-cells. Approximately 1-2 x 10^5 T84 cells (with more than 80% confluence) in DMEM/F12 medium (GIBCO/Invitrogen) were seeded at each well in a 24-well plate. After removing the medium, plate was washed twice with washing buffer, and each well was added with 700 µl DMEM/F12 medium containing 1mM isobutylmethylxanthine (IBMX; phosphodiesterase inhibitor) and incubated at 37 ºC in 5% CO2 for 45 min. Ten ng CT or 2 ng STa toxin (diluted in 150-µl DMEM/F12 medium) was incubated with 150 µl anti-sera (1:50 dilution in DMEM/F12 medium, in triplicate) at room temperature. After 1 h incubation, a mixture of 150 µl CT or STa toxin dilution and 150 ul of diluted anti-sera or anti-fecal sample was added to each well, and the plate was further incubated at 37 ºC in 5% CO_2 for 2 hours. After another wash, the cells were lysed with 0.1M HCl, and then neutralized with 0.1 M NaOH. The cell lysis was collected with a centrifugation at 660 × g for 10 min at room temperature. Resultant supernatants were tested for intracellular cAMP or cGMP levels by following the manufacturer’s protocols.

Statistical analysis Data were analyzed by using the mixed procedure (SAS for windows, version 8; SAS Institute, Cary, N.C.), and Student’s t-test for the different treatment groups was compared. Calculated p values of < 0.05 were regarded as significant.

Results:

Ten strains including one STa recombinant (8330), three STa mutant (8413, 8415, 8417), four LT and STa toxoid chimera strains (8474, 8475, 8552, 8554), a host strain (8227) and a negative control (8331) were constructed for this study (Table 1). Expressed STa proteins were verified and their toxicity and biological activity were confirmed. LT and STa toxoid fusion proteins were purified and used to immunize adult rabbits. Elicited anti-LT and anti-STa antibodies were titrated and examined for activity in neutralizing CT and STa toxins.

Table 1. Escherichia coli strains and plasmids used in the study. A porcine E. coli field isolate G58-2 was used as a parental strain and was transformed with a plasmid pDMS167 expressing 987P fimbria for a host strain (G58/987P). This G58/987P was transformed with a pSTa, a pSTa- mutant, or a chimeric pSTa plasmid for a porcine STa recombinant strain, three STa mutant constructs, and ‘pLT_{192}:$pSTa_{12}$’ and ‘pLT_{192}:$pSTa_{13}$’ fusion constructs. In addition, TOPO 10 cells and TA cloning vectors (Invitrogen) were used for expression and purification of the ‘pLT_{192}:$pSTa_{12}$’ and ‘pLT_{192}:$pSTa_{13}$’ fusion proteins.

Strains	Relevant properties	Plasmid	Reference
G58-2	porcine *E. coli* field isolate		Francis & Willogohs, 1991
04-21018	a porcine ETEC isolate	(F18/STa/STb/Stx2e)	Zhang et al., 2007
8221	LT_{192} construct, 1836-2/pLT_{192}	pLT_{192}/pBR322	Zhang & Francis, submitted
8227	a host strain, G58/987P	pDMS167	this Study
8331	a negative control, G58/987P/pACYC184	pACYC184	this study
8330	pSTa recombinant strain, G58/987P/STa	pSTa/pACYC184	this study
8413	pSTa_{11} mutant, G58/987P/STa_{11}	pSTa_{11}/pACYC184	this study
8415	pSTa_{12} mutant, G58/987P/STa_{12}	pSTa_{12}/pACYC184	this study
8417	pSTa_{13} mutant, G58/987P/STa_{13}	pSTa_{13}/pACYC184	this study
8474	pLT_{192}:pSTa_{12}, G58/987P/LT_{192}:pSTa_{12}	pLT_{192}:pSTa_{12}/pACYC184	this study
8475	pLT_{192}:pSTa_{13}, G58/987P/LT_{192}:pSTa_{13}	pLT_{192}:pSTa_{13}/pACYC184	this study
8552	pLT_{192}:pSTa_{12}, TOPO 10/LT_{192}:STa_{12}	pLT_{192}:pSTa_{12}/ TA vector	this study
8554	pLT_{192}:pSTa_{13}, TOPO 10/LT_{192}:STa_{13}	pLT_{192}:pSTa_{13}/ TA vector	this study

Table 2. PCR primers used for construction of STa recombinant, STa mutants, and 'pLT_{192}:pSTa-toxoid' chimeric strains. To construct the pST recombinant strain, we used primers 184STaSfcI-F and BRSTaEagI-R to amplify porcine *est*A gene and cloned it into pACYC184 (SfcI/EagI sites). Nucleotides in shadow indicated position of a changed amino acid for STa toxoids, and nucleotides in italic are the "Gly-Pro-Gly-Pro" linker. Primers pLT:STa-F and pLT:STa-R are used to fuse porcine *elt*AB (coding LT_{AB} protein) with porcine *est*A (coding STa protein) genes.

Primers	Sequence (5'- '3)	
184EcoRV-F	gtcaggcaccgtgtatgaaat	plasmid pACYC184 sequence at the EcoRV site
pBREagI-R	gtccctgatggtcgtcatct	plasmid pACYC 184 sequence downstream at EagI site
STaSfcI-F	gtgaaacaacctgtaggga	amplify native *estA* gene, underlined is the SfcI site
STaEagI-R	gtggagccggccgaaaca	amplify native *estA* gene, underlined is the EagI site
pSTa_{11K}-F	gaactttgttgtaaacctgcctgt	pairs with pBREagI-R to mutate the 11th amino acid
pSTa_{11K}-R	acaggcaggtttacaacaaagttc	pairs with 184EcoRV-F to mutate the 11th amino acid
pSTa_{12F}-F	ctttgttgtaatttgcctgtgcc	pairs with pBREagI-R to mutate the 12th amino acid
pSTa_{12F}-R	ggcacaggcaaaattacaacaaag	pairs with 184EcoRV-F to mutate the 12th amino acid
pSTa_{13Q}-F	tgttgtaatcctcagtgtgctgga	pairs with pBREagI-R to mutate the 13th amino acid
pSTa_{13Q}-R	tccagcacactgaggattacaaca	pairs with 184EcoRV-F to mutate the 13th amino acid

pLT:STa-F	gcaatcagt *gggccggggccc* atgaacaacttttactgctg	3'end of LT+linker+5'end of STa
pLT:STa-R	gttgttcat gggccccggccc actgattgccgcaattgaattgg	5'end of STa+linker+3'end of LT
$STa_{12}NS$-R	ataacatccagcacaggcaaaatt	STa_{12} mutant without stop codon, for TA cloning
$ST_{13}NS$-R	ataacatccagcacactgagg	STa13 mutant without stop codon, for TA cloning
hLT-F	atgattgacatcatgttgcatatagg	to amplify 'LT_{192}:mSTa' fusion genes, for TA cloning

STa proteins were expressed by STa recombinant and mutant strains. STa proteins were detected in the STa recombinant and all mutant strains. Supernatant samples from overnight-grown 4AA cultures of the recombinant STa (8330), mutant STa_{11} (8413), STa_{12} (8415), STa_{13} (8417), and negative control (8331) were used to compete with 1.25 ng of synthetic STa-ovalbumin conjugates (in 75 ul ELISA dilution buffer, pre-coated in each well) for anti-STa antibodies in a STa competitive ELISA. ELISA results indicated that pre-coated synthetic STa-ovalbumin conjugates bound 30%, 50%, 63%, and 29.2% of the anti-STa serum in competing with STa proteins from 8330, 8413, 8415, and 8417, that differed significantly from the negative control 8331 (97%) (Fig. 1). By referring to the standard curve generating from using a series of STa synthetic peptide samples (0, 0.01, 0.05, 0.10, 0.50, 1.0, 5.0, and 10 ng in 75 µl STa ELISA buffer), mutant STa proteins were produced in a range from 1.3 ng/ml to 13 ng/ml in 8413, 8415, and 8417 strains.

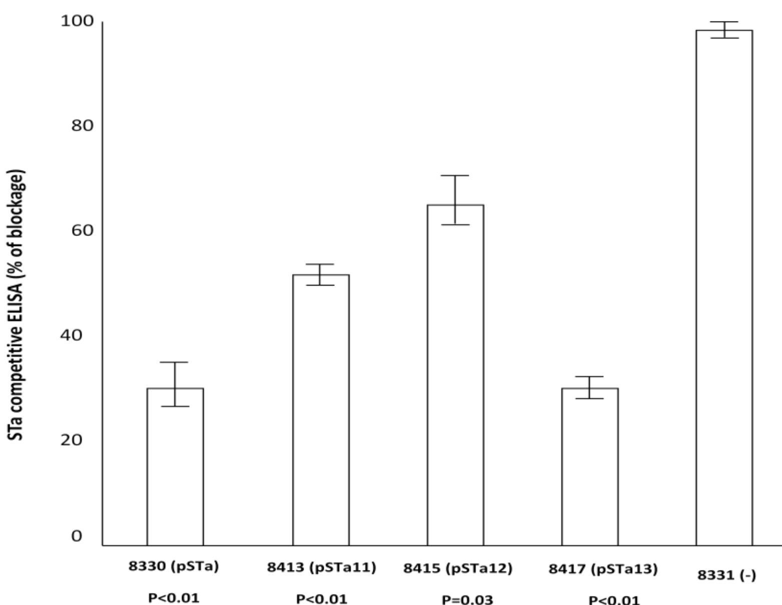


Figure 1. STa competitive ELISA to detect expression of STa protein in STa recombinant and mutant strains 8330 (STa), 8413 (STa_{11}), 8415(STa_{12}) and 8417(STa_{13}). Three mutated *estA* genes were cloned into pACYC184 vector and expressed in G58-2/987p cells. Differences in blocking recognition between coated STa-Ovalbumin conjugates (1.25 ng/well) and anti-STa serum (1:10,000) reflects structure alteration in STa toxoids. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (IgG; 1:10,000) was used as the secondary antibodies. Optical densities were measured at 405 nm.

STa proteins expressed by STa mutant strains are no longer toxic to cause disease. STa proteins expressed in all three mutant strains showed significant reduction in toxicity. Results from cGMP ELISA (acetylated version) indicated that intracellular cGMP concentrations in cells stimulated by 8330, 8413, 8415, 8417, and 8331 culture were 4 ± 0, 0.0185 ± 0.0065, 0.043 ± 0.015, 0.017 ± 0.0015, and 0.012 ± 0.0005 pmole/ml, respectively (Fig. 2). All mutant strains showed low or no stimulation on cGMP levels compared to the recombinant strain, suggesting these mutant STa proteins had toxicity reduced substantially. Statistical analysis indicated that stimulation of cGMP from three mutant STa toxins was significantly lower compared to that from the recombinant STa ($p < 0.01$, $p < 0.01$, $p < 0.01$). Mutant STa_{12} (8415) showed a highest stimulation of cGMP level (43 fmole/ml) and STa_{13} (8417) had the lowest stimulation of cGMP (17 fmole/ml) which was not significantly different from the negative control strain 8331 (12 fmole/ml; p=0.25).

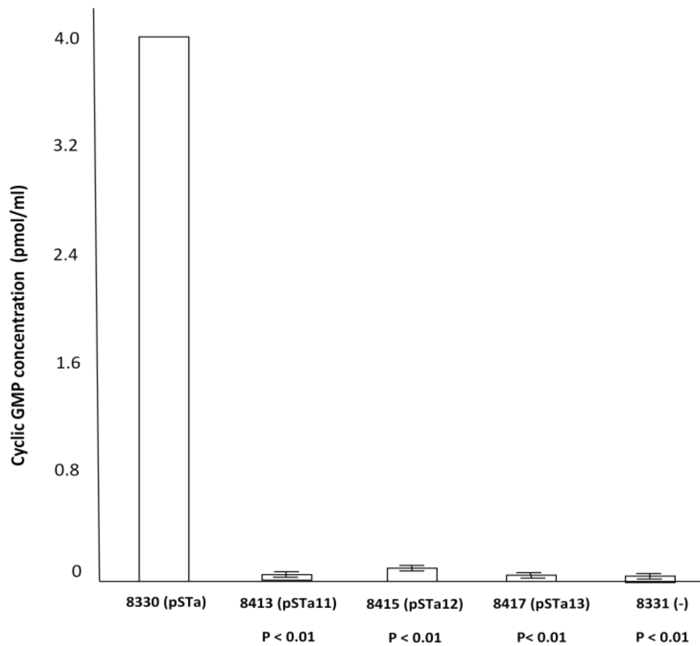


Figure 2. Cyclic GMP ELISA to detect toxicity of STa toxoids. Culture supernatant from STa toxoid strains was used to stimulate T-84 cells for an increase of intracellular cGMP levels by using cGMP EIA kit (Assay Design).

The STa proteins expressed in three mutant strains failed in stimulating fluid secretion in ligated porcine intestines. Ligated loops form a five-day old pig were used to test fluid stimulation for constructs 8330, 8413, 8415, 8417, and the negative control strain 8331. After 8 h incubation, only loops injected with the recombinant STa strain (8330) overnight-grown culture showed fluid accumulation (0.3 g/cm), whereas loops incubated with 8413, 8415 and 8417 mutant strains had 0.02, 0.03, 0.04 g/cm fluid accumulated, respectively. Fluid accumulation in loops incubated with the mutant strains and the negative control was significantly different from that in loops inoculated with the recombinant strain ($p<0.01$) (Fig. 3).

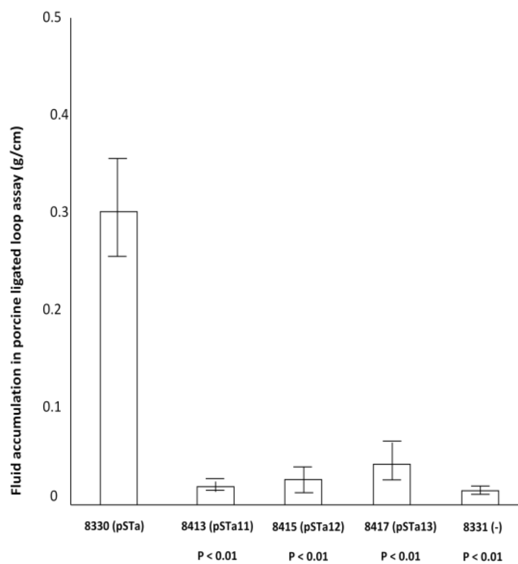


Figure 3. Porcine ligated gut loop assay to detect STa toxoid toxic activity. 2 x 10^9 CFUs of 8330 (STa), 8413 (STa_{11}), 8415(STa_{12}), 8417(STa_{13}) or a negative control 8331(-) strain were incubated in each loop (three repeats). After 8 h incubation, fluid accumulated in each loop was measured, and a ratio of the accumulated fluid (gram) and the loop length (cm) was used as an index.

To test whether expressed STa toxoids were safe to young pigs, we challenged three-day old gnotobiotic pigs that express 987P receptors with each STa mutant strain. During 24 h. post-inoculation, only pigs in the group challenged with the STa recombinant strain developed diarrhea, whereas pigs inoculated with STa mutant strains showed no sign of diarrhea. To affirm all piglets possessing 987P receptors, we collected small intestinal samples from each challenged pig at necropsy to prepare brush border vesicles for testing presence of 987P receptors. Brush border bacterial adherence assay showed all brush borders were positive in binding 987P fimbrial bacteria. In addition, quantitative culture studies showed that colonization of mutant STa constructs ranged from 8.0 x 10^8 to 1.7 x 10^9 CFUs per g of ileum tissue, suggesting all mutant strains were well colonize in small intestines of the challenged pigs.

Porcine LT and STa toxoid fusion proteins enhanced STa immunogenicity. Toxoids $pSTa_{12}$ and $pSTa_{13}$ were selected for constructing fusion proteins. Toxoid $pSTa_{12}$ had the lowest anti-STa antibody recognition but retained a higher toxicity. In contrast, pSTa13 was the best in anti-STa antibody recognition but showed a lower stimulation of intracellular cGMP level in T-84 cells. Both LT and STa toxoids in 'pLT_{192}:$pSTa_{12}$' and 'pLT_{192}:$pSTa_{13}$'fusions were recognized by anti-CT and anti-STa antisera as the Western blot assay detected a band at approximately 15 KDa (the LT_B subunit plus the STa). Anti-STa IgG titers in serum samples from rabbits immunized with purified the 'pLT_{192}:$pSTa_{12}$' and 'pLT_{192}:$pSTa_{13}$' fusion antigens were 3.33 ± 0.23 and 2.59 ± 0.01(in $\log_{10}$), respectively. Anti-STa IgA antibodies were detected at titers of 1.92 ± 0.34 and 1.83 ± 0.17 (in $\log_{10}$; Fig. 4). Anti-STa Se-IgA antibodies were titrated at 1.75 ± 0.14 and 1.26 ± 0.40 in fecal samples of rabbits immunized with 'pLT_{192}:$pSTa_{12}$' and 'pLT_{192}:$pSTa_{13}$'fusion antigens. Anti-LT antibodies (IgG) were detected at high titers (3.33 ± 0.02, 2.71 ± 0.01) (Fig. 4).

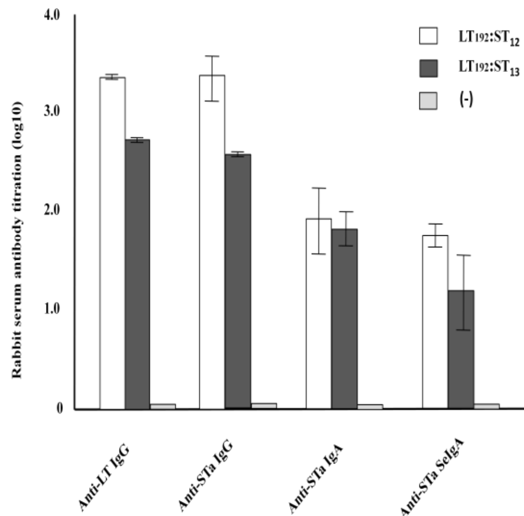
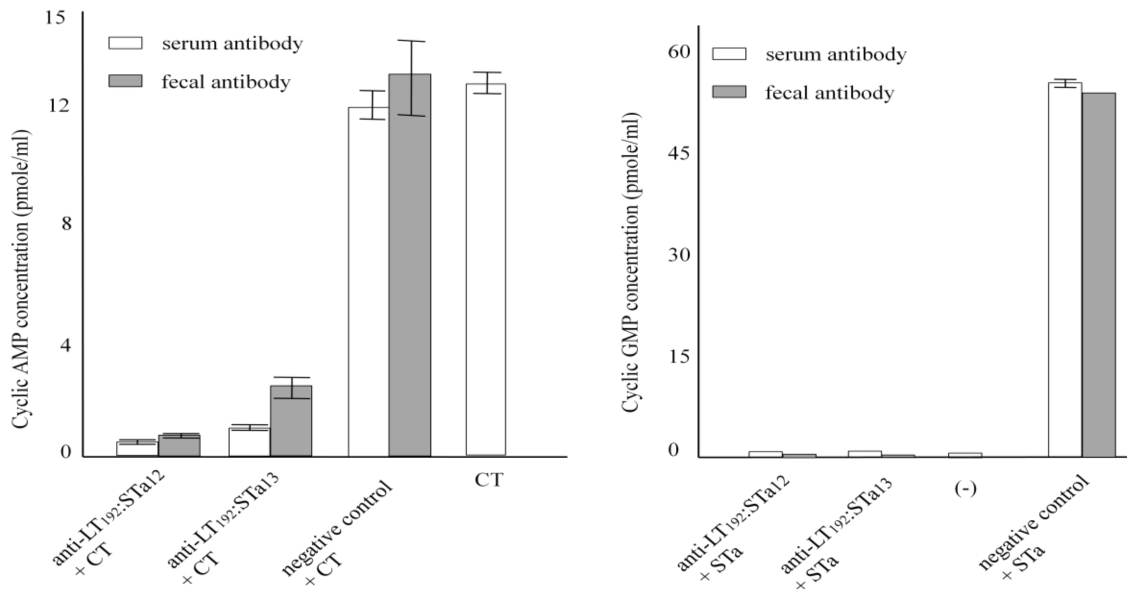


Figure 4. Antibody titration from serum and fecal samples from rabbits immunized with 'pLT_{192}:$pSTa_{12}$' or 'pLT_{192}:$pSTa_{13}$' fusion antigen. The titer (in log10) of anti-LT was assayed in a LT ELISA using purified CT and antigen, and serum samples as the primary antibody. To titer anti-STa antibody, STa ovalbumin-conjugates were used as antigen, and serum and fecal samples were used as the primary antibody. HRP-conjugated IgG and IgA antibodies were used as the secondary. Optical densities greater than 0.4 (after subtracting the background reading) were used to convert to antibody titers (in log10).

Anti-LT and anti-STa antibodies neutralized CT and STa toxins. CT toxin did not stimulated an increase of intracellular cAMP level in T-84 cells after being incubated with serum and fecal samples from rabbits immunized with 'pLT_{192}:$pSTa_{12}$' and 'pLT_{192}:$pSTa_{13}$'fusion antigens (Fig. 5). In contrast, serum or fecal samples from the negative control rabbit did not inhabit CT toxin to increase cAMP levels in T-84 cells. Cyclic AMP concentrations in cells treated with a mixture of CT and anti-'pLT_{192}:$pSTa_{12}$' or anti-'pLT_{192}:$pSTa_{13}$' serum were 0.4 ± 0.01 and 0.52 ± 0.04 pmole/ml, respectively, but the cAMP concentrations in cells treated with CT only and CT mixed with the serum sample from the negative control rabbit were 12 ± 0.25 and 12.7 ± 0.2 pmole/ml, respectively. Similarly, purified STa toxin did not increase intracellular cGMP levels after being incubated with anti-'pLT_{192}:$pSTa_{12}$' and anti-'pLT_{192}:$pSTa_{13}$' serum or fecal samples (Fig. 5). Intracellular cGMP concentration in cells treated with STa mixed with anti-'pLT_{192}:$pSTa_{12}$' or anti-'pLT_{192}:$pSTa_{13}$' antiserum were 0.17 ± 0.005 and 0.16 ± 0.004 pmole/ml. These cGMP levels were significantly lower than those in cells incubated with STa toxin and the serum sample from the negative control rabbit (54 ± 1.0 pmole/ml). Similar results were observed when STa toxin was incubated with anti-fecal antibodies. The cGMP levels in cells after treated with STa toxin and anti-'pLT_{192}:$pSTa_{12}$' or anti-'pLT_{192}:$pSTa_{13}$' fecal antibodies were 0.098 and 0.12 pmole/ml, respectively. Those cGMP levels were not different compared to levels from cells incubated with cell culture medium (0.13 ± 0.03pmole/ml). However, the cGMP concentration in cells treated with STa and the fecal sample from the negative control rabbit was 50 pmole/ml.



Discussion:

Results from this study demonstrated for the first time that porcine STa toxoids can become antigenic when carried by a strongly immunogenic LT toxoid LT_{192}. Rabbits immunized with 'pLT_{192}:$pSTa_{12}$' and 'pLT_{192}:$pSTa_{13}$' fusion antigens produced protective anti-LT and anti-STa antibodies. Since ETEC strains producing STa toxin are equivalent or even more virulent in diarrhea disease than LT-producing ETEC and the anti-LT immunity alone is not sufficiently effective in

Figure 5. Anti-LT and anti-STa antibody neutralization. Serum and fecal samples (1:50 dilution) from rabbits immunized with 'pLT_{192}:$pSTa_{12}$' or 'pLT_{192}:$pSTa_{13}$' fusion antigen were used to neutralize purified CT (10 ng) or STa (2 ng). The mixture was added to T-84 cells to test any increasing of intracellular cGMP (STa; an ELISA kit from Assay Design) or cAMP (CT; an ELISA kit from Invitrogen) levels. Cell culture medium alone was included as a negative control. CT or STa toxin alone, or incubated with a serum or a fecal sample from the negative control rabbit were included for testing its stimulation of cGMP (STa) or cAMP (CT) levels in T-84 cells.

preventing ETEC diarrhea disease (Levine et al., 1979), both STa and LT antigens should be included as essential components in developing effective vaccines against ETEC. However, in contrast to a large and strongly immunogenic LT antigen, STa toxins produced by porcine and human ETEC strains are small and poorly immunogenic (Sears and Kaper, 1996). Therefore, STa alone is unable to elicit anti-STa antibodies in hosts. It has been demonstrated that the human STa protein became immunogenic when it was linked to a carrier protein chemically (Klipstein et al., 1982) or genetically (Sanchez et al., 1986; Guzman-Verduzco and Kupersztoch, 1987; Sanchez et al., 1988; Clements, 1990; Batisson and Der Vatanian, 2000). The genetic fusion approach becomes preferred because of precisely definition of the final products, easy manipulation and low cost (Clement, 1990). When a native human STa gene was genetically fused to a native human *eltA* (coding LT A subunit) or *eltB* (coding LT B subunit) gene, the fusion products were recognized by anti-STa and anti-LT or anti-LTB monoclonal antibodies (Sanchez et al., 1986; 1988). However, these LT_A:STa and LT_B:STa fusion proteins were not tested for STa immunogenicity or toxicity. Clements (1990) generated a LT and STa fusion protein by fusing the hSTa gene at the 3' end of LT_B gene and demonstrated that antibodies arose from mice immunized with this fusion could neutralize native STa toxin.

In contrast to the extensive studies in enhancing hSTa toxin immunogenicity, no study has been conducted to facilitate pSTa immunogenicity and its potential application in developing anti-STa immunity. In addition, previous studies of hSTa antigens either did not address antigen toxicity and potential effects in developing safe vaccines, or suggested that STa toxoid could not stimulate anti-STa immunity even when it was fused to a strongly immunogenic carry

protein. Batisson and Der Vartanian (2000) suggested that only a native STa or STa peptides that retain toxicity were able to stimulate anti-STa antigenicity when they were jointed to the C-terminus of the ClpG major subunit of *E. coli* CS31A fimbriae. A similar fusion protein but with a nontoxic STa protein that had both 13th and 14th amino acids replaced failed in stimulating anti-STa antigenicity (Batisson and Der Vartanian, 2000). Our current study first time demonstrated that STa toxoids are able to facilitate anti-STa antigenicity when they were genetically fused to a pLT_{AB} toxoid. Rabbits immunized with the 'pLT_{192}:$pSTa_{12}$' or 'pLT_{192}:$pSTa_{13}$' fusion antigen produced high titers (in log_{10}) of anti-STa IgG (2.59 – 3.33), IgA (1.83 – 1.92), and Se-IgA (1.26 –1.75). Furthermore, these anti-serum and anti-fecal anti-STa antibodies are capable to neutralize purified STa toxin, thus suggesting arose anti-STa antibodies are protective. Our data were not agree with results from the study by Batisson and Der Vartanian (2000) in which the STa toxoid carried by CS31 major subunit had both the 13th (P→G) and the 14th (A→L) amino acids substituted. As indicated in an early study that after a substitution of alanine to a leucine at the 14th amino acid, the hSTa showed the greatest reduction in toxicity, and the $STa_{14(A \to L)}$ toxoid was three times less toxic than the $STa_{14(A \to Q)}$ (Hirayama, 1995). Substitution of two amino acids made that STa toxoid not only much less toxic, but also certain degree of change in protein structure as it could not be recognized by anti-STa antibodies (Fig. 1 and fig. 2 in Batisson and Der Vartanian, 2000). The STa toxoids, $pSTa_{12(P \to F)}$ and $pSTa_{13(A \to Q)}$, constructed in this study each had only one amino acid substituted, and both toxoids were recognized by anti-STa antibodies, indicating these two toxoids had no major structure alteration occurred. In addition, although either STa toxoid was unable to stimulate fluid secretion in porcine gut loops or an increase of cGMP levels in T-84 cells, both toxoids likely retained very low toxicity, as indicated by Hirayama (1995) that the $hSTa_{12(P \to F)}$ or $hSTa_{13(A \to Q)}$ peptide become biological active when more than a thousand folds of each peptide was used. The retention of very low toxicity and maintaining of proper STa structure likely allowed the STa toxoids constructed in this study had anti-STa antigenicity facilitated when they were carried by a strongly immunogenic protein.

Differed from early studies, we fused a full-length, single amino-acid-substituted, disulfide bond-retained pSTa toxoid to the pLT_{AB} toxoid in this study. Human STa protein retained its toxicity when a native hSTa gene was fused to an hLT_B gene (Guzman-Verduzco and Kupersztoch, 1987; Clements, 1990). Retention of STa toxicity raises safety concerns regarding application in vaccine development. It was reported that hSTa needed to be toxic in order to be antigenicity (Svennerholm and Holmgren, 1992; 1995; Batisson and Vartinian, 2000). However, Sanchez and Holmgren (1988) reported that an hSTa with a disulfide bond disrupted was not toxic, and a fusion protein from this nontoxic hSTa peptide and a CT_B subunit was able to elicit production of antibodies that could be recognized native hSTa protein. In this study, we kept disulfide bonds intact but mutated nucleotides encoding the 11, 12, or 13 amino acid residue of the porcine STa gene. We selected to replace the 11, 12 and 13 amino acids for pSTa toxoid construction because early studies demonstrated that a change of the 12, 13, or 14 amino acid of the hSTa peptide substantially reduced hSTa toxicity (Yamasaki et al., 1988; 1990; Hirayama, 1995). All three constructed pSTa toxoids showed toxicity reduction and they no longer increased cyclic GMP levels in cells nor stimulated fluid secretion in porcine ligated gut loops. Our animal challenge study indicated that *E. coli* constructs expressing these pSTa toxoids are not diarrheagenic to young pigs, suggesting these three STa toxoids could serve as safe antigens. Results from the STa competitive ELISA showed that the $pSTa_{13}$ had least alteration in protein structure as it recognized anti-STa antibodies nearly the same as the native STa protein, whereas the $pSTa_{12}$ perhaps had relatively the greatest structure alteration as it blocked least anti-STa antibodies that bound to the coated STa ovalbumin-conjugates. Cyclic GMP ELISA suggested that $pSTa_{13}$ was least toxic and $pSTa_{12}$ had relative higher toxicity to the T-84 cells. But these results were conflicted with the data from porcine ligated gut loop assay which indicated that loops incubated with the strain expressing $pSTa_{13}$ showed relatively more fluid accumulated. However, Hirayama (1995) demonstrated that minimum effective dose (to be biological active) for $hSTa_{12(N \to K)}$, $hSTa_{13(P \to F)}$ and $hST_{14(A \to Q)}$ are 1300, 1200, and 2090 pmole, respectively. That suggests that a substitution of an alanine to a glutamine of STa resulted in more toxicity reduction. Therefore, we selected the STa_{12} and STa_{13} toxids for fusion protein construction to explore importance of STa toxicity and protein structure in fusion protein eliciting anti-STa antigenicity. But a further study is needed to determine enhancement in antigenicity and vaccine from the $pSTa_{11(N \to K)}$ toxoid. Our results also indicated that the 'pLT_{192}:$pSTa_{12}$' fusion protein facilitated a higher anti-STa antibody titer than the 'pLT_{192}:$pSTa_{13}$' fusion (3.33 vs. 2.59 IgG, 1.92 vs. 1.83 IgA, and 1.75 vs. 1.26 se-IGA, in log10). These results may suggest that toxicity of a STa toxoid indeed play a role in anti-STa antigenicity.

Our study showed that antibodies elicited from fusion antigens of the full-length pLT and pSTa toxoids could neutralize CT and STa toxins. We used CT rather than LT because CT is commercially available. We realized that CT toxin has some differences from LT toxin in antigenicity, however, CT and LT are highly homologous in structure and function. Therefore, we believe that the anti-LT antibodies elicited from immunization of the LT and STa toxoid fusions certainly can neutralize LT toxin as they neutralized CT toxin. To further evaluate host anti-LT and anti-STa immunity,

especially mucosal immunity, induced by immunization of the 'pLT_{192}:$pSTa_{12}$' or 'pLT_{192}:$pSTa_{13}$' antigens and host immunity protection against ETEC infection, we need to conduct animal challenge studies in future. However, results from this study clearly provide helpful information for future development of safe and effective toxoid vaccines against ETEC diarrhea in humans and farm animals.