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Antibodies derived from a toxoid MEFA (multiepitope fusion antigen) show neutralizing activities against heat-labile toxin (LT), heat-stable toxins (STa, STb), and Shiga toxin 2e (Stx2e) of porcine enterotoxigenic *Escherichia coli* (ETEC)

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ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) strains are the main cause of diarrhea in pigs. Pig diarrhea especially post-weaning diarrhea remains one of the most important swine diseases. ETEC bacterial fimbriae including K88, F18, 987P, K99 and F41 promote bacterial attachment to intestinal epithelial cells and facilitate ETEC colonization in pig small intestine. ETEC enterotoxins including heat-labile toxin (LT) and heat-stable toxins type Ia (porcine-type STa) and type II (STb) stimulate fluid hyper-secretion, leading to watery diarrhea. Blocking bacteria colonization and/or neutralizing enterotoxicity of ETEC toxins are considered effective prevention against ETEC diarrhea. In this study, we applied the MEFA (multiepitope fusion antigen) strategy to create toxoid MEFAs that carried antigenic elements of ETEC toxins, and examined for broad antitoxin immunogenicity in a murine model. By embedding STa toxoid STa_{P12F} (NTFYCCELCCNFACAGCY), a STb epitope (KKDLCEHY), and an epitope of Stx2e A subunit (QSYVSSLN) into the A1 peptide of a monomeric LT toxoid (LT_{R192G}), two toxoid MEFAs, 'LT_{R192G}-STb-Stx2e-STa_{P12F}' and 'LT_{R192G}-STb-Stx2e-3xSTa_{P12F}' which carried three copies of STa_{P12F}, were constructed. Mice intraperitoneally immunized with each toxoid MEFA developed IgG antibodies to all four toxins. Induced antibodies showed *in vitro* neutralizing activities against LT, STa, STb and Stx2e toxins. Moreover, suckling piglets born by a gilt immunized with 'LT_{R192G}-STb-Stx2e-3xSTa_{P12F}' were protected when challenged with ETEC strains, whereas piglets born by a control gilt developed diarrhea. Results from this study showed that the toxoid MEFA induced broadly antitoxin antibodies, and suggested potential application of the toxoid MEFA for developing a broad-spectrum vaccine against ETEC diarrhea in pigs.

1. Introduction

Young piglets commonly develop diarrhea, clinical conditions known as neonatal diarrhea and post-weaning diarrhea. Pig neonatal diarrhea and post-weaning diarrhea are caused by pathogenic bacteria and viruses including diarrheal *Escherichia coli*, coronaviruses (transmissible gastroenteritis virus – TGE, and porcine epidemic diarrhea virus – PEDV) and rotaviruses, and continue to be the most important swine diseases (Fairbrother et al., 2005; Harvey et al., 2005; USDA, 2002). Diarrhea results in weight loss, slow growth and acute death, causing substantial economic losses to swine producers in the U.S. and other countries (Haesebrouck et al., 2004; Nagy and Fekete, 1999; Verdonck et al., 2002; Vu-Khac et al., 2007). While neonatal diarrhea can be effectively prevented by passive protection of maternal antibodies, through immunization of pregnant sows; there are no effective prevention strategies against post-weaning diarrhea in weaned pigs. Vaccination is considered the most practical and likely the most effective approach to prevent pig diarrhea. Unfortunately, there are no broadly effective vaccines available to protect young

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pigs against diarrhea particularly post-weaning diarrhea (Melkebeek et al., 2013; Ruan et al., 2011).

E. coli strains have a central role in the etiology of pig diarrhea (Hampson, 1994). Among the types of *E. coli* causing diarrhea in pigs (Nataro and Kaper, 1998), enterotoxigenic *E. coli* (ETEC, viz. a group of *E. coli* strains producing enterotoxins) is by far the most common and important. These ETEC strains produce fimbrial adhesins and enterotoxins. Fimbrial adhesins mediate ETEC bacterial attachment to specific receptors at pig intestinal epithelial cells and facilitate colonization in the pig small intestine (Smith and Linggood, 1971), playing an essential role in initiating the disease. But it is the enterotoxins that disrupt fluid homeostasis in pig small intestinal epithelial cells to cause fluid and electrolyte hyper-secretion, leading to diarrhea (Nataro and Kaper, 1998). Fluid hyper-secretion results in the loss of some of the mucin layer and disruption of tight junctions of microvilli that further enhance bacterial colonization and worsen diarrheal disease (Berberov et al., 2004; Glenn et al., 2009; Zhang et al., 2006). Therefore, fimbrial adhesins and enterotoxins are the key virulence determinants of ETEC diarrhea, and have been long targeted for prevention strategy development.

Fimbrial adhesins expressed by ETEC strains causing diarrhea in pigs are K88 (F4), F18, K99 (F5), 987P (F6), and F41 (F7) (Awad-Masalmeh et al., 1982; Casey et al., 1992; Frydendahl, 2002; Moon, 1990; Moseley et al., 1986; Nagy et al., 1977; Zhang et al., 2007). Toxins produced by porcine ETEC strains are porcine-type heat-labile toxin (pLT; which is homologous to but differs from the LT of ETEC causing human diarrhea—hLT), heat-stable toxin type Ia (pSTa or porcine-type STa), heat-stable toxin type II (STb), Shiga toxin type 2e (Stx2e), and enteroaggregative heat-stable toxin type 1 (EAST1) (Frydendahl, 2002; Lee et al., 1983; Moon et al., 1980; Nakazawa et al., 1987; Osek, 1999; Zhang et al., 2007). Clinical observations and epidemiological studies revealed that a typical ETEC strain expresses one and occasionally two fimbrial adhesins and one, two or more toxins (Francis, 2002; Frydendahl, 2002; Zhang et al., 2007). Laboratory experimental studies demonstrated that an ETEC strain expressing one fimbrial adhesin and LT, STb, or STa enterotoxin causes diarrhea in young pigs (Berberov et al., 2004; Erume et al., 2008; Zhang et al., 2006; Zhang et al., 2008). Unlike LT and STa or STb, Stx2e is a member of the Shiga toxin family, and itself is thought to be primarily associated with Edema disease in young pigs (Bertschiner and Gyle, 1994). But as Stx2e is frequently found in ETEC strains expressing LT and/or ST toxins (Zajacova et al., 2012; Zhang et al., 2007), it was also targeted for ETEC diarrhea prevention. In contrast, *E. coli* strains expressing fimbriae and EAST1 are found not associated with diarrhea in young pigs (Ruan et al., 2012; Zajacova et al., 2013). It is thought that a vaccine blocking attachment from all ETEC fimbrial adhesins to host receptors and/or eliminating enterotoxicity of ETEC toxins to host epithelial cells would be able to effectively protect against ETEC diarrhea in pigs and humans (Boedeker, 2005; Walker, 2005; Zhang and Sack, 2012).

However, developing effective vaccines against ETEC continues to be difficult because ETEC strains are divergent. Different ETEC bacteria express immunologically heterogeneous fimbrial adhesins and distinctive enterotoxins. To overcome this challenge, innovative antigen preparation approaches are needed. The recently developed MEFA (multiepitope fusion antigen) strategy allowed us to include antigenic elements (epitopes) from multiple human ETEC virulence factors into a single recombinant protein to induce broadly protective antibody responses (Ruan et al., 2015; Ruan et al., 2014a). We also found recently that peptides (or toxoids) derived from mutated LT and STa toxins are safe antigens and LT-STa genetic fusions induce protective antibodies against both LT and ST toxins (Liu et al., 2011; Ruan et al., 2014b; Zhang et al., 2010). By applying the MEFA, toxoid, and genetic fusion approaches, we should be able to include antigenic elements from all porcine ETEC toxins into a single antigen and to develop a safe and broadly effective antitoxin vaccine.

In this study, we applied the toxoid antigens and the MEFA strategy to create LT_{R192G}-STb-Stx2e-STa_{P12F} toxoid MEFAs, examined the toxoid MEFA for broad antitoxin antigenicity, and explored potential application of the toxoid MEFA in development of a broadly effective antitoxin vaccine against ETEC-associated diarrhea in pigs.

2. Methods

2.1. Bacteria strains and plasmids

The *E. coli* strains and plasmids used in this study are listed in Table 1. Genomic DNA of porcine *E. coli* field strains 3030-2 (K88/LT/STb/STa) (Zhang et al., 2006) and 9168 (F18/Stx2e) (Zhang et al., 2007), and STb recombinant strain 8020 (Zhang et al., 2006) were used for PCR amplification of the *eltAB* genes (LT), the *estA* gene (STa), the Stx2e A subunit gene and the *estB* gene (STb), respectively. LT mutant strain 8221 (Zhang et al., 2010) and STa mutant strain 8415 (Zhang et al., 2010) were used for LT_{R192G} and STa_{P12F} gene amplification. Strains 9168 and 8020 were also used in Vero cell test to detect antibody neutralizing activity against Stx2e toxin and STb toxin. Recombinant ETEC strains, 8819 (987P/LT), 8816 (987P/STb) and 8823 (987P/STa) were used in piglet challenge studies. Vector pET28α (Novagen, Madison, WI) was used to clone and to express the toxoid MEFA genes, and vector pMAL-p5X (New England Biolabs, Ipswich, MA) was used to clone the *estB* gene and the Stx2e A subunit gene for expression of MBP (maltose binding protein)-STb and MBP-Stx2e fusion proteins. *E. coli* strains BL21 (GE Healthcare, Piscataway, NJ) and DH5α (Promega, Madison, WI) were used to express toxoid MEFA and MBP fusion proteins. Recombinant *E. coli* strains were cultured in Lysogeny broth (LB) supplemented with kanamycin (30 μg/ml) or ampicillin (100 μg/ml).

2.2. ETEC toxoid MEFA gene construction

B-cell epitopes from the LT A1 peptide, STb toxin, and the Stx2e A subunit were predicted with web-based software (Odorico and Pellequer, 2003; Saha and Raghava, 2007). Full-length STa toxoid STa_{P12F} gene was first embedded into LT_{A1} of a modified LT gene coding a monomeric LT_{R192G} (one A subunit and one B subunit into a single peptide); nucleotides coding the STb epitope and the Stx2e A subunit epitope were sequentially embedded into the LT_{R192G}-STa_{P12F} chimeric gene by using SOE (splice overlapping extension) PCR as we described previously (Liu et al., 2011; Ruan et al., 2014b; Zhang et al., 2010).

To embed nucleotides coding the STa_{P12F} mature peptide into the LT_{A1} of LT_{R192G}, two PCR products were generated and overlapped. The first PCR product was amplified with primers LT_{192}NheI-F3 (5-GTTTGCTAGCAATGGCGACAAATTATAC-′3; NheI site underlined) and LT_{192}-STa_{P12F}-R (5′-*GGCAAAATTACAACAAAGTTCACAGCAGTAAAATGTGTTGTT*TTCATCAATCACACCAAAATTAACACGATACCA-′3; nucleotides of STa_{P12F} in italic, with mutated nucleotides in shade; nucleotides of LT_{A1} underlined). The second PCR product was amplified with primers LT_{192}-STa_{P12F}-F (5′-*CTTTGTTGTAATTTTGCCTGTGCTGGATGTTAT*ATAGCTCCGGCAGAGGATGGTTACAGA-′3; nucleotides of STa_{P12F} in italic, with mutated nucleotides in shade; nucleotides of LT_{A1} underlined) and LT_{192}BamHI-R1 (5′-GCGTGGATCCCTACTAGTTTTCCATACT-′3; BamH1 site underlined). LT_{R192G} plasmid p8221 was used as DNA templates for both PCRs. Overlapping two PCR products (through the STa complementary nucleotides of primers) generated a LT_{R192G}-

STa_{P12F} chimeric gene. Digested with NheI and BamH1 (Biolabs), this LT_{R192G}-STa_{P12F} toxoid chimeric gene was cloned into pET28α. Resultant plasmids were introduced into DH5α competent cells for a LT_{R192G}-STa_{P12F} recombinant strain.

Similar to the insertion of STa_{P12F} into the LT_{A1} of LT_{R192G}, nucleotides coding the STb epitope and the Stx2e A subunit epitope were sequentially inserted into the A1 fragment of the LT_{R192G}-STa_{P12F} chimeric gene. To insert the STb epitope to create chimeric gene LT_{192}-STb-STa_{P12F}, we overlapped two PCR products: one was amplified with primers T7-F (5′-TAATACGACTCACTATAGGG-′3) and STb-LT_AChim-R (5′-*ATGTTCACACA-GATCTTTTTT*GCCGGTTTGTGTTCCTCTCGCGTG-′3; nucleotides of STb epitope in italic, and nucleotides of LT_{A1} underlined), and the other was amplified with primers LT_A-STbChim-F (5′-*GATCTGTGTGAACATTAT*GTTTCCACTTCTCTTAGTTTGAGAAGT-′3; nucleotides of STb epitope in italic, and nucleotides of LT_{A1} underlined) and T7-R (5′-TGCTAGTTATTGGTCAGGGGT-′3), with plasmid pLT_{R192G}-STa_{P12F} as the DNA template.

Insertion of nucleotides coding the Stx2e A subunit epitope into chimeric gene LT_{192}-STb-STa_{P12F} was completed by overlapping another two PCR products. One was amplified with T7-F and Stx2e-LT_A-R (5′-*CGAAGATACATAACTTTGTTG*TATATACTGTCCTGCTAAGT-GAGC-′3; nucleotides of Stx2e A subunit epitope in italic, and nucleotides of LT_{A1}underlined), and the other was amplified with primers LT_A-Stx2e-F (5′-*AGTTATGTATCTTCGTTAAAT*TATATCGTTA-TAGCAAATATGTTT-′3; nucleotides of Stx2e A subunit epitope in italic, and nucleotides of LT_{A1}underlined) and T7-R, with plasmid pLT_{R192G}-STb-STa_{P12F} as the DNA template. The overlapped PCR product was amplified with primers T7-F and T7-R, digested with NheI and BamH1, and cloned into pET28α.

To enhance anti-STa antigenicity, two additional copies of STa_{P12F} gene were fused at the 5′ end and the 3′ end of the LT_{R192G}-STb-Stx2e-STa_{P12F} gene for a toxoid MEFA which carries three copies of STa toxoid STa_{P12F} (LT_{R192G}-STb-Stx2e-3xSTa_{P12F}).

2.3. Expression and detection of ETEC toxoid MEFA protein

Expression of toxoid MEFA proteins was examined in a standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Toxoid MEFA recombinant strains were cultured at 37 °C in 200 ml Lysogeny broth (LB) supplemented with kanamycin (30 μg/ml). Bacteria in the overnight grown culture, after OD_{600} reached 0.6, were induced with 0.1 mM isopropyl-1-thio-β-D-galactoside (IPTG) and incubated for 4 more hours. Induced bacteria were collected and used for total protein extraction in B-PER (bacterial protein extraction reagent in phosphate buffer; Pierce, Rockford, IL). B-PER extracted proteins (with a majority of proteins as inclusion bodies; in denaturing buffer) were used for further extraction of 6 × His-tagged toxoid MEFA proteins to a purity of greater than 90% with Ni-nitrolotriacetic acid agarose by following the manufacturer's protocol (QIAGEN, Valencia, CA). The 6 × His tagged toxoid MEFA proteins were refolded with a Novagen protein refolding kit (Novagen).

The 6xHis-tagged protein (10 μg) of either toxoid MEFA recombinant strain was examined using 12% SDS-PAGE gels with rabbit anti-CT (1:3,000; Sigma, St. Louis, MO), anti-STa (1:5,000; protein A-purified, gift from Dr. DC Robertson, Kansas State Univ.), anti-STb (1:1,000; National Animal Disease Center, Ames, Iowa), and anti-Stx2e serum (1:3,000; National Animal Disease Center). IRDye-labeled goat anti-rabbit IgG (1:5,000; LI-COR, Lincoln, NE) and LI-COR Odyssey premium infrared gel imaging system (LI-COR) were used to detect the toxoid MEFA proteins. Protein purity was assessed using standard Coomassie blue staining as previously described (Ruan et al., 2014a).

2.4. Mouse immunization

Ten 8-week-old female BALB/c mice (Charles River Laboratories International, Inc., Wilmington, MA) in a group were each immunized intraperitoneally with 200 μg of 6 × His-tagged toxoid

Table 1
Escherichia coli strains and plasmid used in the study.

Strains	Relevant properties	Sources
BL21	*B F*$^-$, *omp*T, *hsd*S (r_B^-, m_B^-), *gal,dcm.*	GE Healthcare
DH5α	*fhu*A2, Δ(*arg*F-*lac*Z), U169, *pho*A, *gln*V44, φ80, Δ(*lac*Z)M15, *gyr*A96, *rec*A1, *rel*A1, *end*A1, *thi*-1,*hsd*R17	Promega
3030–2	Field isolate, K88/LT/STb/STa	Zhang et al., 2006
8020	K88/STb (pRAS1 in 1836–2)	Zhang et al., 2007
8221	LT_{R912G} mutant strain	Zhang et al., 2010
8415	STa_{P12F} mutant strain	Zhang et al., 2010
8221	LT_{R912G} mutant strain	Zhang et al., 2010
8415	STa_{P12F} mutant strain	Zhang et al., 2010
8221	LT_{R912G} mutant strain	Zhang et al., 2010
8415	STa_{P12F} mutant strain	Zhang et al., 2010
8221	LT_{R912G} mutant strain	Zhang et al., 2010
8415	STa_{P12F} mutant strain	Zhang et al., 2010
8221	LT_{R912G} mutant strain	Zhang et al., 2010
8415	STa_{P12F} mutant strain	Zhang et al., 2010
9168	04-13812 field isolate, F18/Stx2e	Zhang et al., 2007
8823	987P/STa challenge strain	Zhang et al., 2010
Plasmids		
pET28α		Novagen
pMAL-p5X		New England Biolabs
pET/LT_{R192G}-STa_{P12F}	LT_{R192G}-STa_{P12F} fusion in pET28α at NheI/BamH1	this study
pET/LT_{R192G}-STb-STa_{P12F}	LT_{R192G}-STb-STa_{P12F} fusion in pET28α at NheI/BamH1	this study
pET/LT_{R192G}-STb-Stx2e-STa_{P12F}	LT_{R192G}-STb-stx2e-STa_{P12F} fusion in pET28α at NheI/BamH1	this study
pET/LT_{R192G}-STb-Stx2e-3xSTa_{P12F}	LT_{R192G}-STb-stx2e-3xSTa_{P12F} fusion in pET28α at NheI/BamH1	this study
pMAL-STb	MBP-STb fusion in pMAL-p5X	this study
pMAL-Stx2eB	MBP-Stx2eA fusion in pMAL-p5X	this study

MEFA protein 'LT_{R192G}-STb-Stx2e-STa_{P12F}' or 'LT_{R192G}-STb-Stx2e-3xSTa_{P12F}' (in 200 μl 0.02 M Tris–HCl protein buffer), in an equal volume of Freund complete adjuvant (Sigma). Immunized mice received two booster injections in a two-week interval, at the same dose of the primary but with Freund incomplete adjuvant. Ten mice immunized with 200 μl 0.02 M Tris–HCl, with 200 μl Freund complete adjuvant (primary) or incomplete adjuvant (boosters) served as the control. Serum samples collected before the primary and 10 days after the final booster were stored at −80 °C until use. On day 38, all mice were anesthetized with CO_2 and exsanguinated. The mouse study complied with the Animal Welfare Act according to National Research Council guidelines (National Research Council, 1996), and was approved by the South Dakota State University Institutional Animal Care and Use Committee and was supervised by the South Dakota state veterinarian.

2.5. Mouse serum antitoxin IgG antibody titration

Serum of each immunized or control mouse was examined for anti-LT, anti-STa, anti-STb and anti-Stx2e IgG antibodies in ELISAs. To titrate anti-LT and anti-STa IgG antibodies, we coated 96-well microtiter plates with cholera toxin (CT, an LT homologue which has been commonly used as the coating antigen to titrate anti-LT antibodies, Sigma; 100 ng per well of Immulon 2HB plates) or STa-ovalbumin conjugates (10 ng per well of Costar plates) as we described previously (Liu et al., 2011; Ruan et al., 2014b; Zhang et al., 2010).

To titrate anti-STb and anti-Stx2e IgG antibodies, we prepared MBP (maltose binding protein)-STb and MBP-Stx2eA fusion proteins and used them as coating antigens. The STb gene was PCR amplified with primers STbNcoII-F (5′-GACCTTTCATCCATGGGCTCTACACAATCAAATAAAAAAGAT-′3; Nco1 site underlined) and STbBamH1-R (5′-GACTTTAGAGGATCCTTAGCATCCTTTTGCTGCAACCAT-′3; BamH1 site underlined), with STb plasmid p8020 (pRAS1) as templates. The Stx2e A subunit gene was amplified with primers Stx2eNco1-F (5′-GACCTTTCATCCATGGGCTTACTGGGTTTTTCTTCGGTATCC-′3; Nco1 site underlined) and Stx2eBamH1-R (5′-GACTTTAGAGGATCCCACGTCTTCCGGCGTCATCGTATA-′3; BamH1 site underlined) with total DNA of field strain 9168 (F18/Stx2e) as templates. PCR amplified STb gene and Stx2e A subunit gene were digested with Nco1 and BamH1, and ligated into pMAL-p5x (BioLabs) for expression of MBP-STb and MBP-Stx2eA fusion proteins. Recombinant fusion proteins MBP-STb and MBP-Stx2eA expressed in *E. coli* DH5α were extracted with the pMAL™ protein fusion and purification system by following the manufacturer's protocol (BioLabs). Extracted MBP-STb and MBP-Stx2eA fusion proteins were refolded with a Novagen protein refolding kit (Novagen), verified in Western blot with anti-STb and anti-Stx2e antiserum respectively, and were used to coat Immulon 2HB plates (100 ng per well) to titrate anti-STb and anti-Stx2e IgG antibodies in ELISAs.

2.6. Antitoxin antibody neutralization

Pooled serum samples from the mice immunized with MEFA LT_{R192G}-STb-Stx2e-3xSTa_{P12F} and the control mice were examined for *in vitro* antibody neutralization activities against LT, STa, STb, and Stx2e. EIA cAMP and cGMP kits (Assay Design, Ann Arbor, MI) and T-84 cells were used to measure neutralizing activity of mouse serum antibodies against LT and STa toxins, as we described previously (Liu et al., 2011; Ruan et al., 2014b; Zhang et al., 2010). Briefly, the serum sample (30 μl) pooled from the immunized group or the control group was incubated with 2 ng STa toxin (gift from Dr. DC Robertson, Kansas State Univ.; diluted in 150 μl Dulbecco modified Eagle medium [DMEM]/F-12 medium) or 10 ng CT (Sigma; in 150 μl DMEM/F-12) for 1 hour at room temperature. Incubated serum/toxin mixture was brought to 300 μl with DMEM/F-12 and added to a confluent monolayer of T-84 cells (10^5 cells per well, in 700 μl DMEM/F-12) pre-treated with 1 mM IBMX (3-isobutyl-1-methylaxanthine; Sigma). After incubation of 1 h (STa for cGMP) or 3 h (CT for cAMP) at 37 °C in a CO_2 incubator, T-84 cells were gently washed and then lysed. Cell lysates were collected and were measured for intracellular cAMP or cGMP levels (pmole/ml) using a cAMP or cGMP ELISA kit by following manufacturer's protocol (Assay Design).

For antibody neutralization activity against STb toxicity, bacterial culture filtrates of strain 8020 (STb+) and Vero cells (ATCC; CCL-81) were examined for cytotoxic activity. Vero cells were seeded in a 24-well plate and cultured to reach full confluence. The volume of 8020 filtrates causing over CD50 of Vero cells was pre-determined and used for the *in vitro* neutralization assay. Mouse serum sample, at 150 μl, 100 μl, 50 μl or 25 μl, pooled from the immunized mice or the control mice was used to be pre-mixed with 300 μl 8020 culture filtrates. Each serum/filtrates mixture was added to Vero cells (in 700 μl Eagle's Minimum Essential Medium). After incubation in a 37 °C CO_2 incubator for 1 h, cells were examined microscopically. The highest dilution that prevented bacteria filtrates from causing CD50 of Vero cells was considered antibody neutralization titer.

To examine antibody neutralization activity against Stx2e toxin, Vero cell test was carried out based on published protocols (Matsuura et al., 2009; Smith and Scotland, 1993), with modification. A volume of filtrates of Stx2e strain 9168 that caused CD50 of Vero cells was pre-determined. That volume (100 μl) of filtrates of 9168 overnight grown culture was mixed with binary diluted mouse serum sample, 50 μl, 25 μl, 12.5 μl, 6.3 μl, 3 μl, or 1.5 μl, pooled from the immunized or the control group. Each mixture was added to Vero cells (confluent monolayer) in a well of a 24-well tissue culture plate (in a final volume of 1 ml with culture medium) and incubated in a CO_2 at 37 °C for 3 days. Cells were examined microscopically daily for cell death (round up) and detachment from wells of the tissue culture plates. Vero cells cultured in cell medium alone or with 100 μl bacterial filtrates were used as references.

2.7. Pig immunization and challenge studies

Gilts that had no ETEC diarrhea record and had not been vaccinated were used for this study. Gilt pre-screened without pre-existing antibodies to ETEC toxins were raised in the university swine unit. A pregnant gilt was intramuscularly immunized with 500 μg recombinant 'LT_{R192G}-STb-Stx2e-3xSTa_{P12F}' (in 500 μl PBS) and 5 μg double mutant LT adjuvant (dmLT, in 50 μl PBS; provided by Walter Reed Army Institute of Research, Silver Spring, MD) 8 weeks before farrowing, and received a booster 4 weeks later. Another gilt IM injected with 500 μl PBS was used as the control. After 24 h suckling, piglets born by the immunized mother and the control mother were orally inoculated with 8819 (2.5×10^9 CFUs), 8816 (2.5×10^9 CFUs) and 8823 (2.5×10^9 CFUs). Challenged piglets were examined every 3–4 h during 24 h post-inoculation. No piglets were challenged with a Stx2e strain. Pig immunization and challenge studies were approved and supervised by Kansas State University IACUC.

2.8. Statistical analysis

Data generated from this study were analyzed using SAS for Windows, version 8 (SAS Institute, Cary, NC). Results were expressed as means and standard deviations. Nonparametric Mood's Median Test or Kruskal–Wallis Median Test was carried

out using SigmaXL (Kitchener, ON, Canada) at 2-sided and 95% confidence to assess differences at results of antibody titration (OD readings or the serum dilution that gave an OD >0.3 above the background) and antibody neutralization (cAMP or cGMP levels, pmole/ml) studies between the immunized group and the control group. Calculated p values of <0.05 indicated that differences were significant, when treatments were compared with a two-tailed distribution and two-sample equal or unequal variance.

3. Results

3.1. The constructed toxoid MEFA carried epitopes of STb and Stx2e A subunit, and STa toxoid STa_{P12F}

By substituting an *in silico* predicted surface-exposed but less antigenic epitope of the monomeric LT_{R192G} (138–154 amino acids of the LT A1 peptide) with the full-length mature peptide of STa_{P12F} toxoid, a 'LT_{R192G}-STa_{P12F}' toxoid chimeric gene was generated. Toxoid STa_{P12F} was reported to induce neutralizing anti-STa antibodies in rabbits after being genetically fused at the C-terminus of monomeric LT_{R192G} peptide (Zhang et al., 2010). This 'LT_{R192G}-STa_{P12F}' was served as the template for subsequent insertions of a STb epitope and a Stx2e epitope. Peptide 'KKDLCEHY' was *in silico* predicted as the only epitope from the poorly immunogenic STb toxin, and 'QSYVSSLN' was among the most antigenic epitopes from the Stx2e A subunit. Replacing nucleotides coding the 52–59 amino acids of the LT_{A1} in the 'LT_{R192G}-STa_{P12F}' chimeric gene with nucleotides coding the STb 'KKDLCEHY' epitope resulted in 'LT_{R192G}-STb-STa_{P12F}' gene. Substituting nucleotides coding the 77–83 amino acids of the LT_{A1} of 'LT_{R192G}-STb-STa_{P12F}' with nucleotides coding the Stx2e A subunit epitope 'QSYVSSLN' yielded the 'LT_{R192G}-STb-Stx2e-STa_{P12F}' gene (Fig. 1A). Accordingly, recombinant strains, 8778 (LT_{R192G}-STa_{P12F}), 9137 (LT_{R192G}-STb-STa_{P12F}), and 9161 (LT_{R192G}-STb-Stx2e-STa_{P12F}) were generated (Table 1).

Fusing two additional STa_{P12F} toxoids at the N-terminus and the C-terminus of the 'LT_{R192G}-STb-Stx2e-STa_{P12F}' created the second toxoid MEFA, recombinant strain 9403 (LT_{R192G}-STb-Stx2e-3xSTa_{P12F}) (Table 1). DNA sequencing revealed that nucleotide fragments coding the STa_{P12F} toxoid, and epitopes of STb and Stx2e A subunit stayed in a correct reading frame.

Expression of the 'LT_{R192G}-STb-Stx2e-STa_{P12F}' or 'LT_{R192G}-STb-Stx2e-3xSTa_{P12F}' toxoid MEFA protein was verified in Western blot using anti-CT, anti-STa, anti-STb, and anti-Stx2e antiserum. A protein of approximately 37–40 kDa, an expected molecule weight of the denatured his-tagged toxoid MEFA 'LT_{R192G}-STb-Stx2e-

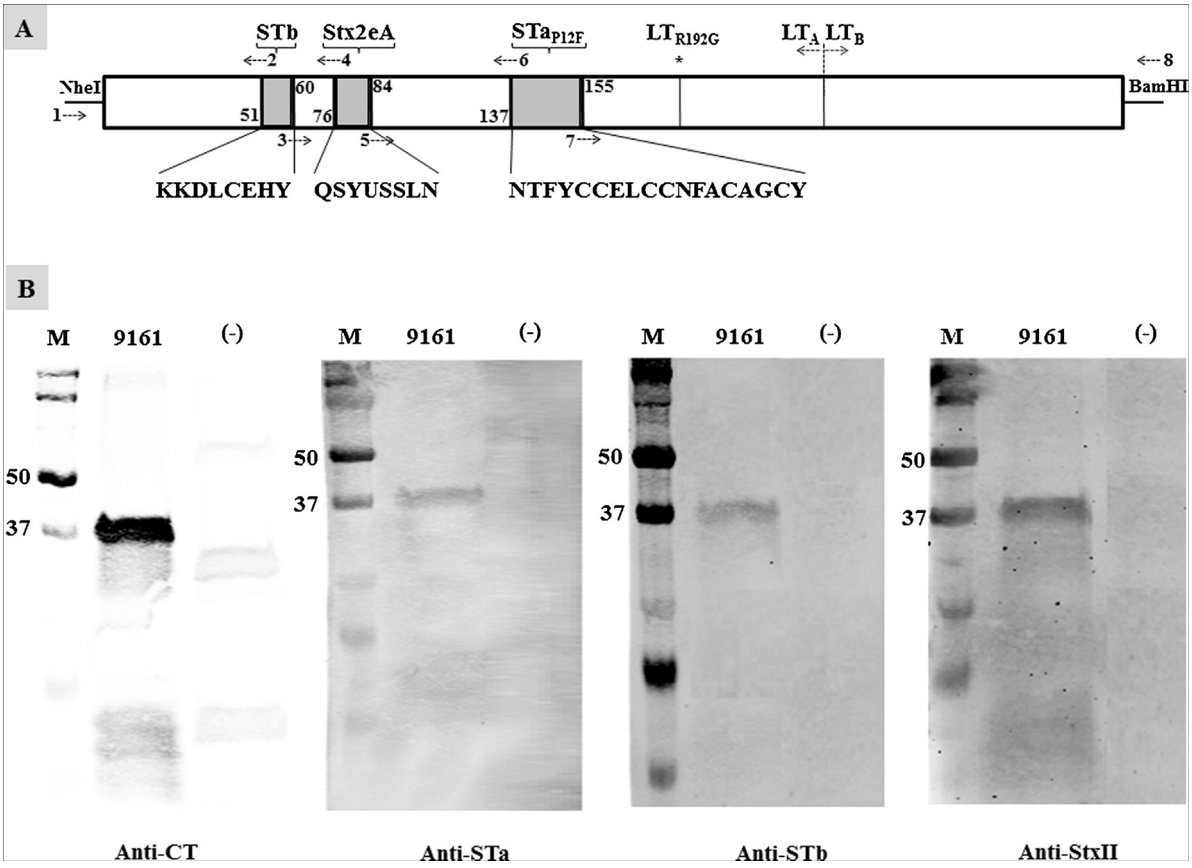

Fig. 1. Construction and detection of LT_{R192G}-STb-Stx2e-STa_{P12F} toxoid MEFA. (A) Schematic illustration of the toxoid MEFA gene. Nucleotides coding STb epitope (KKDLCEHY), Stx2e A subunit epitope (QSYVSSLN), and full-length STa toxoid STa_{P12F} substituted nucleotides coding the 52–59, 77–83, and 138–154 amino acids of the A1 peptide of the monomeric LT toxoid LT_{R192G} to generate a toxoid MEFA gene using splicing overlap extension (SOE) PCRs. PCR primers: 1, T7-F; 2, STb-LT_AChim-R; 3, LT_A-STbChim-F; 4, Stx2e-LT_A-R; 5, LT_A-Stx2e-F; 6, LT_{192}-STa_{P12F}-R; 7, LT_{192}-STa_{P12F}-F; 8, T7-R. Two PCRs using primers LT_{192}NheI-F3 and 6, primers 7 and LT_{192}BamH1-R1 constructed the LT_{R192G}-STa_{P12F} chimeric gene initially. The second set of two PCRs using primers 1 and 2, primers 3 and 8 to insert the STb epitope into the LT_{R192G}-STa_{P12F} toxoid chimeric gene, and the third set of two PCRs with primers 1 and 4, primers 5 and 8 to insert the Ste2e A subunit epitope into the LT_{R192G}-STb-STa_{P12F} chimeric gene. (B) Western blots to show detection of the toxoid MEFA protein with rabbit anti-CT (1:3000; Sigma), anti-STa (1:5000), anti-STb (1:1000), and anti-Stx2e (1:3000) antiserum, with IRDye-labeled goat-anti-rabbit IgG (1:5000; LI-COR) as the secondary antibody. Lane M is the protein marker (in kilo Daltons; Precision Plus Protein Pre-stained standard; Bio-Rad), 9161 represented proteins extracted from the toxoid MEFA recombinant strain, and (−) indicated proteins extracted from *E. coli* BL21 host strain.

STa_{P12F}', was detected from strain 9161 by anti-CT, anti-STa, anti-STb, and anti-StxII antiserum, respectively (Fig. 1B). A protein of approximately 40 kDa was detected from strain 9403 (LT_{R192G}-STb-Stx2e-3xSTa_{P12F}) using the anti-STa and anti-LT antibodies. No proteins of the similar sizes were detected by the same anti-serum from the host *E. coli* BL21 strain.

3.2. Toxoid MEFA proteins were immunogenic

Both toxoid MEFAs were unable to stimulate cAMP or cGMP in T-84 cells, indicating a lack of LT or STa enterotoxicity (data not shown). Mice immunized with toxoid MEFA 'LT_{R192G}-STb-Stx2e-STa_{P12F}' developed strong anti-LT, anti-STb and anti-Stx2e IgG antibody responses (Fig. 2). Anti-LT, anti-STb and anti-Stx2e IgG titers (in $\log_{10}$) were 3.75 ± 0.34, 3.37 ± 0.42 and 3.6 ± 0.53, respectively in the immunized mice. These titers were significantly greater than those of the control mice (anti-LT, 0.1 ± 0.1, anti-STb, 0.42 ± 0.61, and anti-Stx2e, 1.26 ± 0.08; $p = 0.000$ from Mood's Median test).

Anti-STa IgG antibody response were detected at a very low titer (0.45 ± 0.21) in the serum of the mice immunized with 'LT_{R192G}-STb-Stx2e-STa_{P12F}' (Fig. 2), but still significantly differed from the response in the control mice ($p = 0.003$ of Mood's Median test). Mice immunized with toxoid MEFA 'LT_{R192G}-STb-Stx2e-3xSTa_{P12F}', which included three copies of the STa_{P12F} toxoid, had anti-STa IgG antibody titers (in $\log_{10}$) detected at 0.87 ± 0.66 from serum samples (Fig. 3).

3.3. Mouse serum anti-LT and anti-STa antibodies exhibited in vitro neutralizing activities against CT and STa toxins

The pooled serum sample of the immunized mice showed neutralizing activities against CT (Fig. 4) and STa (Fig. 5). The cAMP level in T-84 cells incubated with 10 ng CT and 30 μl pooled serum of the immunized mice was 15.84 ± 3.40 pmole/ml; the cAMP level in the T-84 cells incubated with the same amount of CT but the pooled serum of the control mice was significantly greater, 41.2 ± 0 pmole/ml ($p < 0.05$ in Mood's Median test).

The cGMP level in the T-84 cells incubated with 2 ng STa and 30 μl pooled serum of the immunized mice was 3.45 ± 0.16 pmole/ml. The cGMP level in the T-84 cells incubated with 2 ng STa but the 30 μl pooled serum sample of the control mice was significantly greater (8.32 ± 0.96 pmole/ml; $p < 0.01$ in Mood's Median test).

3.4. Serum antibodies showed in vitro neutralizing activity against STb and Stx2e cytotoxicity

The pooled serum sample of the immunized mice reduced STb cytotoxicity to Vero cells (Fig. 6). Vero cells incubated with 300 μl 8020 filtrates alone (Fig. 6B), or 300 μl filtrates and 150 μl pooled serum of the control mice (Fig. 6C), were shown over 50% cell death or detachment. Cells incubated with 300 μl 8020 filtrates and 150 μl (1:7.7 dilution) pooled serum of the immunized mice remained normal (Fig. 6D). When serum of the immunized mice was reduced to 25 μl (1:41 dilution), cells started showing 50% detachment (Fig. 6F).

Stx2e toxicity Vero cell test showed 50% of the Vero cells became rounded and detached after incubation with 100 μl filtrates of Stx2e strain 9168 overnight grown culture (Fig. 7B) or in the addition of 50 μl pooled serum sample of the control mice (Fig. 7C). But cells remained normal when incubated with 100 μl 9168 filtrates and 50 μl pooled serum sample (1:20 final dilution, in 1 ml cell culture medium) of the immunized mice (Fig. 7D). Only when less than 12.5 μl pooled serum of the immunized mice was used (a final dilution of 1:80), over 50% of the cells become dead and detached (Fig. 7F).

3.5. Piglets born by the immunized mother were protected against ETEC challenge

Immunized gilt and piglets born by the immunized gilt had anti-LT, anti-STb, anti-STa and antiStx2e antibodies detected

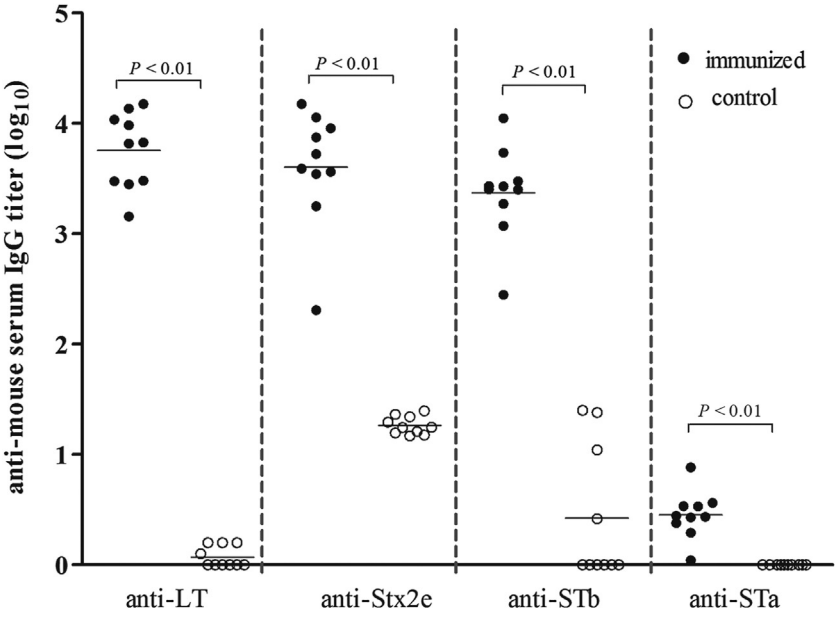

Fig. 2. Mouse serum anti-LT, anti-Stx2e, anti-STb and anti-STa IgG antibody titers (in $\log_{10}$). Anti-LT, anti-Stx2e, anti-STb and anti-STa IgG antibodies in serum samples of the mice immunized with 'LT_{R192G}-STb-Stx2e-STa_{P12F}' MEFA and the control mice (10 mice per group) were titrated in ELISAs using CT (100 ng per well of 2HB plates; coating antigens), MBP-Stx2eA fusion (100 ng per well of 2HB plates), MBP-STb fusion (100 ng per well of 2HB plates), and STa-ovalbumin conjugates (10 ng per well of Costar plates), respectively. HRP-conjugated goat anti-mouse IgG (1:5000 for anti-LT, anti-Stx2e and anti-STb; 1:3000 for anti-STa) was used the secondary antibodies. Antibody titers were calculated from the highest dilution of a serum sample that produced an ELISA OD of >0.3 (above the background), and expressed in a scale of $\log_{10}$. Each dot represented an IgG titer from a mouse, and the bar indicated the mean titer of the group. The *p* value indicated difference of the IgG titers between the immunization group and the control group.

(Table 2). After challenged with 8819 (LT), 8816 (STb) and 8823 (STa) recombinant ETEC strains together, all 6 piglets born by the control mother developed severe diarrhea and showed sign of dehydration, whereas 6 out 7 piglets born by the immunized mother remained healthy. Only one piglet born by the immunized mother showed mild diarrhea. No antibodies specific to LT, STa, STb or Stx2e were detected in the control gilt, piglets born by the control gilt, or serum samples of gilts prior to primary immunization.

4. Discussion

Since ETEC strains expressing any one, two, or more than two toxins cause diarrhea in neonatal and post-weaning pigs, an effective ETEC antitoxin vaccine need to induce antibodies protecting against LT, STa and STb. Results from the present study indicated that a toxoid MEFA that carried antigenic elements or epitopes of four ETEC toxins induced anti-LT, anti-STa, anti-STb and anti-Stx2e antibodies. Moreover, antibodies derived from the immunized mice showed *in vitro* neutralization activities against toxicity of all four toxins, and antibodies derived from the immunized gilt showed protection against LT/STa/STb ETEC infection. These results suggest toxoid MEFA LT_{R192G}-STb-Stx2e-3xSTa_{P12F} can potentially be an antigen for developing a protective antitoxin vaccine against ETEC associated diarrhea in young pigs.

STa toxoid STa_{P12F} when was fused to the C-terminus of the LT toxoid LT_{R192G} induced strongly protective anti-STa IgG and IgA antibody responses in IM immunized rabbits (Zhang et al., 2010). Data from the current study, however, showed that MEFA LT_{R192G}-STb-Stx2e-STa_{P12F}, which had the same STa toxoid but embedded inside the A1 peptide of the LT_{R192G}, induced only mild IgG antibody response in the IP immunized mice. That suggested the STa toxoid may not be placed at an optimal position in this toxoid MEFA, negatively affecting its antigen presentation and thus anti-STa antigenicity. Knowing that additional copies of STa toxoid enhanced toxoid fusion anti-STa antigenicity (Zhang et al., 2013), we constructed toxoid MEFA 'LT_{R192G}-STb-Stx2e-3xSTa_{P12F}' to carry three copies of the STa_{P12F}. Mice immunized with LT_{R192G}-STb-Stx2e-3xSTa_{P12F} developed similar levels of anti-LT, anti-STb and anti-Stx2e IgG antibody responses compared to the mice immunized with toxin MEFA 'LT_{R192G}-STb-Stx2e-STa_{P12F}' (data not shown), but a greater anti-STa IgG antibody titer (Fig. 3). Whether STa_{P12F} is the optimal STa toxoid for toxoid MEFAs to induce protective anti-STa antibodies will need to be further examined. Human-type STa toxoid STa_{N12S} is suggested an optimal STa toxoid for LT-STa toxoid fusions in inducing neutralizing anti-STa antibodies (Ruan et al., 2014b). But the human-type STa and the porcine-type STa show antigenicity differences, whether the

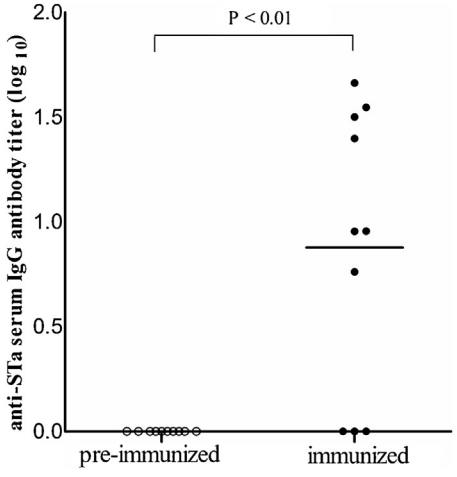

Fig. 3. Anti-STa IgG titers (in log_{10}) in serum samples of mice immunized with toxoid MEFA 'LT_{R192G}-STb-Stx2e-3xSTa_{P12F}'. Anti-STa IgG antibodies in the serum samples of mice before the immunization and after the immunization (10 mice in the group) were titrated in ELISA using STa-ovalbumin conjugates (10 ng per well of Costar plates; coating antigen) and HRP-conjugated goat anti-mouse IgG (1:3000; the secondary antibodies). Each dot represented a mouse IgG titer, and the bar indicated the mean titer of the treatment.

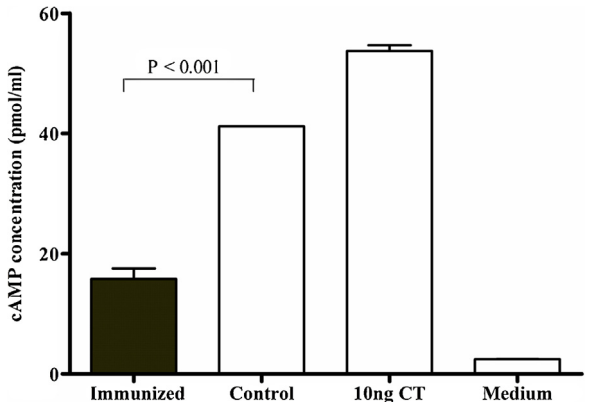

Fig. 4. Mouse serum *in vitro* antibody neutralizing activity against CT. Intracellular cyclic AMP levels in T-84 cells measured with an EIA cAMP ELISA kit (Assay Design) showed anti-LT antibody neutralization activity. Neutralizing antibodies prevent CT from stimulating intracellular cAMP in T-84 cells, resulting in a low cAMP level (pmole/ml). The pooled serum sample of the mice immunized with 'LT_{R192G}-STb-Stx2e-3xSTa_{P12F}' or the control mice (30 μl in total) was incubated with 10 ng CT toxin (in 150 μl) for 1 h at room temperature, the serum/toxin mixture was brought to 300 μl and added to T-84 cells (in 700 μl cell culture medium). Intracellular cAMP levels in cells were measured after 3 h incubation. Ten ng CT without serum was used as a positive control, and medium (cell culture medium; without toxin or serum) was used as the background reference. Columns and bars indicated mean cAMP levels and standard deviations. The *p* value of Mood's Median test indicated difference of antibody neutralizing activity against CT between the immunization group and the control group.

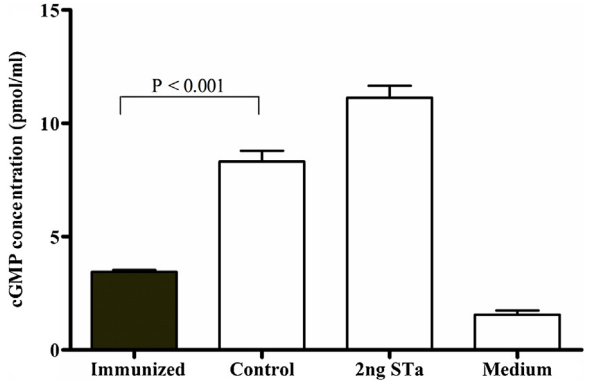

Fig. 5. Mouse serum *in vitro* antibody neutralizing activity against STa toxin. Intracellular cyclic GMP levels in T-84 cells measured with an EIA cGMP ELISA kit (Assay Design) showed anti-STa antibody neutralization activity. Neutralizing antibodies prevent STa toxin from stimulating intracellular cGMP in T-84 cells, resulting in a low cGMP level (pmole/ml). The pooled serum sample of the mice immunized with 'LT_{R192G}-STb-Stx2e-3xSTa_{P12F}' or the control mice (30 μl in total) was incubated with 2 ng STa toxin (in 150 μl) for 1 h at room temperature, the serum/toxin mixture was brought to 300 μl and added to T-84 cells (in 700 μl cell culture medium). Intracellular cGMP levels in cells were measured after 1 h incubation. Two ng STa without serum was used as a positive control of enterotoxicity and medium (cell culture medium; without toxin or serum) was used as the background reference. Columns and bars indicated mean cGMP levels and standard deviations. The *p* value of Mood's Median test indicated difference of antibody neutralizing activity against STa between the immunization group and the control group.

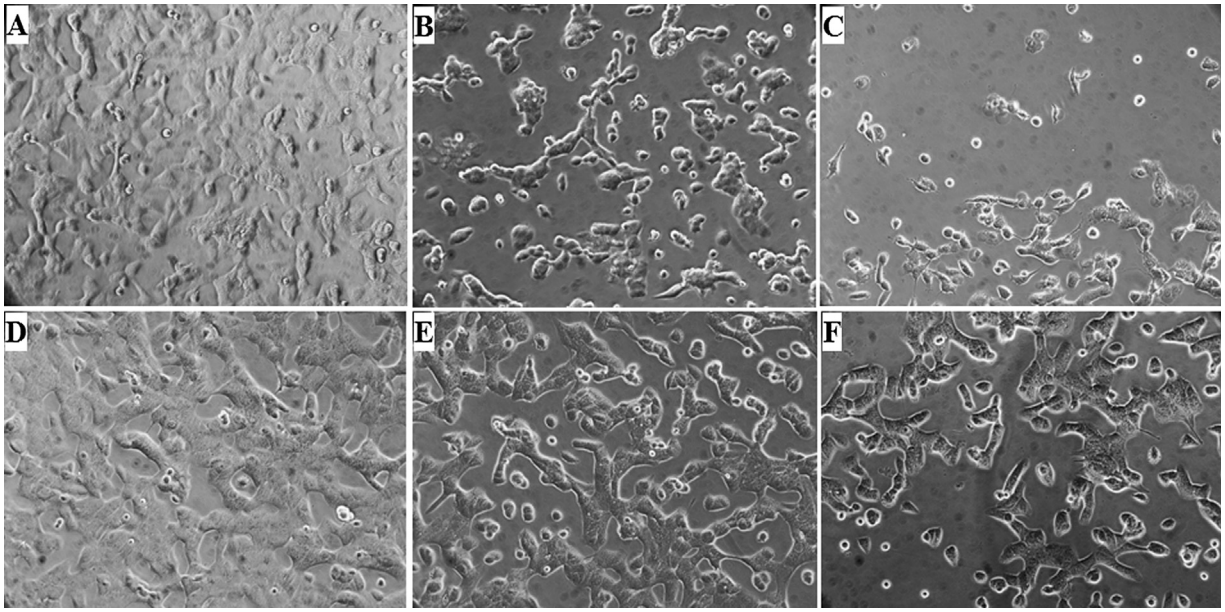

Fig. 6. Mouse serum antibody neutralization activity against STb toxin. A: Normal Vero cells grown in cell culture medium. B: Vero cells (in 700 μl culture medium) incubated with 300 μl 8020 (STb) overnight grown culture filtrates, showing over 50% cell death and detachment. C: Vero cells incubated 300 μl 8020 (STb) filtrates pre-mixed with 150 μl pooled serum of the control mice. D: Vero cells incubated with 300 μl 8020 (STb) filtrates pre-mixed with 150 μl pooled serum of the mice immunized with 'LT_{R192G}-STb-Stx2e-3xSTa_{P12F}' MEFA. E: Vero cells incubated with 300 μl 8020 (STb) filtrates premixed with 50 μl pooled serum of the immunized mice. F: Vero cells incubated with 300 μl 8020 (STb) filtrates and 25 μl pooled serum of the immunized mice.

analogue porcine-type STa_{N11S} exhibits better antigenic property needs to be further examined. Nevertheless, antibodies induced by the LT_{R192G}-STb-Stx2e-3xSTa_{P12F} showed neutralizing activity against STa toxin, and protected suckling piglets against LT/STb/STa ETEC infection.

LT_{R192G}-STb-Stx2e-STa_{P12F} and LT_{R192G}-STb-Stx2e-3xSTa_{P12F} generated in this study may be used to develop antitoxin subunit vaccines, as a step for further development of practical and economical vaccines to prevent diarrhea in pigs. Unlike live attenuated vaccines, subunit vaccines are not optimal for preventing post-weaning diarrhea in pigs because of a higher production cost and the demand for professional training and labor in parenteral immunization to large numbers of individual pigs. Helpfully, the monomeric 'LT_{R192G}-STb-Stx2e-STa_{P12F}' MEFA had

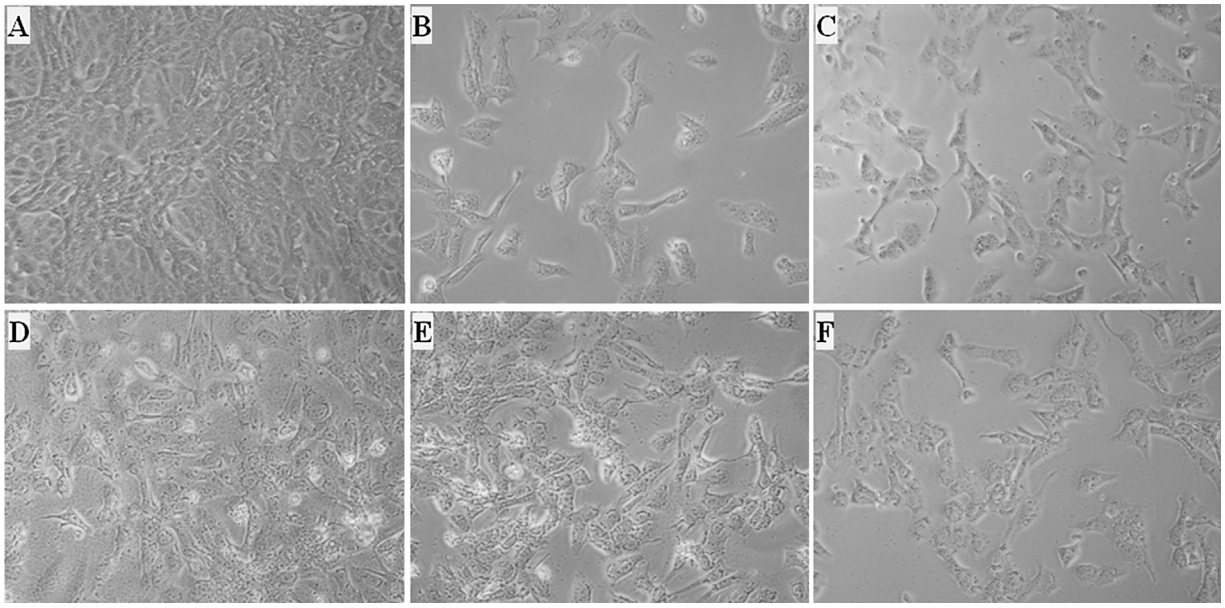

Fig. 7. Mouse serum antibody neutralization activity against Stx2e in Vero cell test. A: Vero cells grow in normal cell culture medium, showing normal cell growth. B: Vero cells incubated with 100 μl of 9168 (F18/Stx2e) strain overnight grown culture filtrates, showing over 50% cell death. C: Vero cells incubated with 100 μl 9168 strain filtrates pre-mixed with 50 μl serum pooled from the control mice. D: Vero cells incubated with 100 μl 9168 strain filtrates pre-mixed with 50 μl serum pooled from the immunized mice. E: Vero cells incubated with 100 μl 9168 strain filtrates and 25 μl serum pooled from the immunized mice. F: Vero cells incubated with 100 μl 9168 strain filtrates and 12.5 μl serum pooled from the immunized mice.

Table 2
Anti-LT, –STb, –Stx2e and anti-STa IgG and IgA antibody titers (log_{10}) detected in the immunized or control sows and the piglets born by the immunized or control mothers.

Anti-LT antibodies								
Sows							Piglets born by	
Serum IgG		Colostrum IgA		Colostrum IgG			Serum IgG	
Immunized	Control	Immunized	Control	Immunized	Control		Immunized	Control
3.40 ± 0.00	0 ± 0	2.6 ± 0.04	0 ± 0	3.32 ± 0.01	0 ± 0		4.15 ± 0.14	0 ± 0
Anti-STb antibodies								
Sows							Piglets born by	
Serum IgG		Colostrum IgA		Colostrum IgG			Serum IgG	
Immunized	Control	Immunized	Control	Immunized	Control		Immunized	Control
3.96 ± 0.03	0 ± 0	2.4 ± 0.02	0 ± 0	3.32 ± 0.04	0 ± 0		3.5 ± 0.12	0 ± 0
Anti-Stx2e antibodies								
Sows							Piglets born by	
Serum IgG		Colostrum IgA		Colostrum IgG			Serum IgG	
Immunized	Control	Immunized	Control	Immunized	Control		Immunized	Control
3.90 ± 0.02	0 ± 0	2.2 ± 0.01	0 ± 0	3.33 ± 0.05	0 ± 0		3.5 ± 0.12	0 ± 0
anti-STa antibodies								
Sows							Piglets born by	
Serum IgG		Colostrum IgA		Colostrum IgG			Serum IgG	
Immunized	Control	Immunized	Control	Immunized	Control		Immunized	Control
1.86 ± 0.01	0 ± 0	0.78 ± 0.01	0 ± 0	1.8 ± 0.01	0 ± 0		1.98 ± 0.10	0 ± 0

Note: The standard deviations of IgG and IgA antibody titers of the immunized sow and the control sow were from triplicate sampling.

the STa toxoid, the STb epitope, and the Stx2e epitope embedded at the A1 peptide of LT_{R192G}. This chimeric gene can be modified to express a LT-like holotoxin-structured toxoid MEFA protein. As we demonstrated previously (Ruan and Zhang, 2013), by simply inserting back the nucleotides coding the native leading signal peptides of the LT_A subunit gene (*eltA*) and the LT_B subunit gene (*eltB*), and also the cistron gene structure between the two LT subunit genes, a monomeric LT gene can be reversed to a LT-like gene coding a LT-like holotoxin-structured protein. Since the native LT_B gene expresses the LT_B subunit and forms LT_B pentamer independently, a modified 'LT_{R192G}-STb-Stx2e-STa_{P12F}' fusion protein can form LT_B pentamer and binds to GM1 receptors in pig small intestine. If expressed by a nonpathogenic *E. coli* strain, the modified LT-like-structured 'LT_{R192G}-STb-Stx2e-STa_{P12F}' toxoid MEFA could be used to develop a live oral vaccine against porcine ETEC diarrhea. Even for the 'LT_{R192G}-STb-Stx2e-3xSTa_{P12F}' toxoid MEFA, if all three copies of STa_{P12F} or a different STa toxoid are embedded at the LT A1 peptide, for an example, at the N- and C-terminus of the A1 peptide, the monomeric 'LT_{R192G}-STb-Stx2e-3xSTa_{P12F}' can also be converted to a holotoxin-structured protein and expressed by an *E. coli* or a *Salmonella* strain for a live ETEC vaccine candidate.

Double mutant LT, dmLT ($LT_{R192G/L211A}$) was used as adjuvant for pig immunization. This dmLT has been explored as a safe and effective mucosal adjuvant in human vaccine development. We recently showed that dmLT can be an effective adjuvant in parenteral immunization (data not shown). Adjuvant dmLT included in current pig immunization also served as an antigen to supplement the toxoid MEFA in inducing protective anti-LT antibodies. Future studies with a different adjuvant can conclusively assess protection from anti-LT antibodies derived from this toxoid MEFA. Additionally, studies will be needed to optimize the dose of dmLT adjuvant and also the dose of toxoid MEFA immunogen.

Future studies to assess better the potency of this toxoid MEFA in ETEC vaccine development will be needed. The present study examined only antigen immunogenicity, *in vitro* antitoxin antibody neutralization and maternal antibody passive protection against ETEC infection. Unlike commercially available kits that were used to measure antibody neutralizing activities against LT or STa, we currently do not have standard assays to measure anti-STb and anti-Stx2e antibody neutralizing activities. In this study, we adopted the Vero cell cytotoxicity assay to assess antibody neutralization activities against STb and Stx2e. Future studies to use purified STb or Stx2e toxin instead of bacterial culture filtrates or to adopt a Shiga toxin quantitative microtiter assay (Gentry and Dalrymple, 1980) may measure better anti-Stx2e or anti-STb antibody neutralization activity. In addition, immunizing neonatal piglets and challenging weaned piglets with the LT+, STb+, and STa + ETEC strains separately will be needed to assess protective efficacy against each toxin, post-weaning diarrhea, and also perhaps Stx2e associated edema disease as described by Oanh et al. (2012). Moreover, large scaled studies are required to evaluate efficacy of candidate vaccines derived from this toxoid MEFA. Results from this study, nevertheless, indicate the toxoid MEFAs can potentially serve as antigens for multivalent vaccines against ETEC associated diarrhea, and may suggest the MEFA strategy may be a useful platform for developing broadly protective vaccines against other heterogeneous pathogenic strains or isolates.

5. Conclusion

STa toxoid peptide, STb and Stx2e-A subunit epitopes of porcine enterotoxigenic *E. coli* (ETEC), the main cause of porcine neonatal diarrhea and post-weaning diarrhea, can be embedded into a LT toxoid monomer. Mouse serum antibodies derived against the toxoid MEFA showed neutralizing activities against all four toxins.

Pigs IM immunized with LT-STb-Stx2e-3xSTa_{P12F} MEFA developed antibodies specific to each toxin, and maternal passive antibodies protected born piglets against infection of a mixture of a LT+, a STa+ and a STb+ strains. These results suggested that a toxoid MEFA may potentially serve as an antigen for vaccine development against porcine ETEC diarrhea.

Conflict of interest

The authors declaim no conflict of interests from this study.

Acknowledgements

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