Neutralizing Anti-Heat-Stable Toxin (STa) Antibodies Derived from Enterotoxigenic *Escherichia coli* Toxoid Fusions with STa Proteins Containing N12S, L9A/N12S, or N12S/A14T Mutations Show Little Cross-Reactivity with Guanylin or Uroguanylin

Qiangde Duan,[a] Jiachen Huang,[a] Nan Xiao,[a] Hyesuk Seo,[a] (ID) Weiping Zhang[a]

[a]Department of Diagnostic Medicine/Pathobiology, Kansas State University College of Veterinary Medicine, Manhattan, Kansas, USA

ABSTRACT Heat-stable toxin (STa)-producing enterotoxigenic *Escherichia coli* (ETEC) strains are a top cause of moderate-to-severe diarrhea in children from developing countries and a common cause of travelers' diarrhea. Recent progress in using STa toxoids and toxoid fusions to induce neutralizing anti-STa antibodies has accelerated ETEC vaccine development. However, concern remains regarding whether the derived anti-STa antibodies cross-react with STa-like guanylin and uroguanylin, two guanylate cyclase C (GC-C) ligands regulating fluid and electrolyte transportation in human intestinal and renal epithelial cells. To further divert STa from guanylin and uroguanylin structurally and antigenically and to eliminate anti-STa antibody cross-reactivity with guanylin and uroguanylin, we mutated STa at the 9th (leucine), 12th (asparagine), and 14th (alanine) residues for the double and triple mutants $STa_{L9A/N12S}$, $STa_{L9A/A14H}$, $STa_{N12S/A14T}$, and $STa_{L9A/N12S/A14H}$. We then fused each STa mutant (three copies) to a monomeric heat-labile toxin (LT) mutant ($mnLT_{R192G/L211A}$) for the toxoid fusions $3 \times STa_{L9A/N12S}-mnLT_{R192G/L211A}$, $3 \times STa_{L9A/A14H}-mnLT_{R192G/L211A}$, $3 \times STa_{N12S/A14T}-mnLT_{R192G/L211A}$, and $3 \times STa_{L9A/N12S/A14H}-mnLT_{R192G/L211A}$; examined each fusion for anti-STa immunogenicity; and assessed the derived antibodies for *in vitro* neutralization activity against STa toxicity and for cross-reactivity with guanylin and uroguanylin. Mice subcutaneously immunized with each fusion protein developed anti-STa antibodies, and the antibodies derived from $3 \times STa_{N12S}-mnLT_{R192G/L211A}$, $3 \times STa_{L9A/N12S}-mnLT_{R192G/L211A}$, or $3 \times STa_{N12S/A14T}-mnLT_{R192G/L211A}$ prevented STa from the stimulation of intracellular cGMP in T-84 cells. Competitive enzyme-linked immunosorbent assays (ELISAs) showed that guanylin and uroguanylin hardly blocked the binding of anti-STa antibodies to the coated STa-ovalbumin conjugate. These results indicated that antibodies derived from $3 \times STa_{N12S}-mnLT_{R192G/L211A}$, $3 \times STa_{L9A/N12S}-mnLT_{R192G/L211A}$, or $3 \times STa_{N12S/A14T}-mnLT_{R192G/L211A}$ neutralized STa and had little cross-reactivity with guanylin and uroguanylin, suggesting that these toxoid fusions are suitable antigens for ETEC vaccines.

IMPORTANCE Enterotoxigenic *Escherichia coli* (ETEC) strains are a leading cause of children's diarrhea and travelers' diarrhea. Currently, there is no licensed vaccine against ETEC diarrhea. One key challenge is to identify safe antigens to induce antibodies neutralizing the key STa without cross-reacting with guanylin and uroguanylin, two important ligands controlling homeostasis in human intestinal and renal epithelial cells. In this study, we generated nontoxic fusion antigens that induced antibodies that neutralize STa enterotoxicity *in vitro* and do not cross-react with guanylin or uroguanylin. These fusions have become the preferred antigens for the development of ETEC vaccines to potentially prevent the deaths of hundreds of thousands of young children and hundreds of millions of diarrheal cases each year.

KEYWORDS ETEC, enterotoxigenic *Escherichia coli*, antibody cross-reactivity, diarrhea, guanylin, uroguanylin

Enterotoxigenic *Escherichia coli* (ETEC) bacteria that produce heat-stable toxin (STa) type Ib (hSTa [human-type STa] or STh), alone or together with heat-labile toxin (LT), are among the top five causes of moderate-to-severe diarrhea in children less than 5 years of age in developing countries (1) and the most common cause of diarrhea in children and adults traveling from developed countries to regions where ETEC is endemic (2–4). Currently, there is no licensed vaccine for ETEC-associated children's diarrhea or travelers' diarrhea. One key challenge in ETEC vaccine development is the inability to use safe STa antigens to induce neutralizing anti-STa antibodies (5, 6). However, recent studies demonstrated that a nontoxic STa mutant (toxoid; 1 to 3 copies) genetically fused to a monomeric LT toxoid (mnLT) (a peptide consisting of one LT_A subunit mutant and one LT_B subunit, to serve as the LT antigen and a carrier to facilitate STa antigenicity) induced antibodies that neutralized STa and LT enterotoxicity (7–10). Among those nontoxic STa-mnLT fusions, $3\times STa_{N12S}$-$mnLT_{R192G/L211A}$ (previously named $3\times STa_{N12S}$-dmLT [10]), which carried three copies of the STa toxoid STa_{N12S} and one copy of the monomeric LT mutant $mnLT_{R192G/L211A}$ (with the LT_A subunit mutated at residues 192 and the 211), was considered optimal in inducing neutralizing anti-STa antibodies (10, 11). Moreover, antibodies derived from the toxoid fusion $3\times STa_{N12S}$-$mnLT_{R192G/L211A}$ prevented STa-positive (STa^+) ETEC diarrhea in a pig challenge model (12), suggesting that $3\times STa_{N12S}$-$mnLT_{R192G/L211A}$ is a leading ETEC vaccine antigen (13).

However, potential cross-reactivity between anti-STa antibodies and guanylin or uroguanylin remains a concern (14). Guanylin and uroguanylin are structurally and functionally similar to STa (15–17) (Fig. 1). Like STa, heat-stable guanylin and uroguanylin regulate water and electrolyte homeostasis in intestinal epithelial cells by activating the guanylate cyclase C (GC-C) pathway and controlling intracellular cGMP levels (15, 18–20). Guanylin- or uroguanylin-regulated Cl^- and HCO_3^- secretion and Na^+ absorption in host epithelial cells can be altered by cross-reactive anti-STa antibodies, potentially leading to adverse health consequences, including hypernatremia.

STa toxoids that induce antibodies that neutralize STa without cross-reacting with guanylin and uroguanylin are preferred for ETEC vaccine development. These STa toxoids would likely have mutations at certain residues to further divert them from guanylin and uroguanylin in structure and antigenicity. Toxoid fusion $3\times STa_{N12S}$-$mnLT_{R192G/L211A}$ carrying the STa toxoid STa_{N12S} induced strongly neutralizing anti-STa antibodies (10–12). However, anti-STa antibodies derived from this toxoid fusion have not been examined for cross-reactivity with guanylin or uroguanylin. Presumably, STa_{N12S} would be separated further from uroguanylin or guanylin if this toxoid has an additional residue(s) mutated, although such STa mutants potentially have altered STa antigenic properties and may induce antibodies not specific to native STa and thus would not neutralize STa enterotoxicity.

In this study, we mutated STa at the 9th, 12th, and 14th residues for STa double or triple mutants; genetically fused each STa mutant (three copies) to the monomer LT mutant $mnLT_{R192G/L211A}$ (a single peptide of one LT_A subunit mutated at residues 192 and 211 and one LT_B subunit); and examined each toxoid fusion for anti-STa immunogenicity. Furthermore, the induced anti-STa antibodies were examined for neutralization activity against STa toxicity and for antibody cross-reactivity with guanylin and uroguanylin.

RESULTS

Production of four genetic fusions carrying three copies of an STa double or triple mutant and one copy of a monomeric LT mutant ($mnLT_{R192G/L211A}$). Guanylin, which has been reported to have no cross-reactivity with anti-STa antibodies (16), differs from uroguanylin (which showed some cross-reactivity with anti-STa antibodies) at the 3rd, 6th, 8th, 9th, and 10th residues (Fig. 1). The noncysteine residues at the 3rd
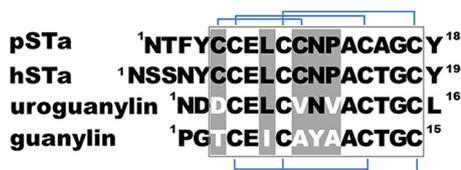
FIG 1 Amino acid sequences of mature porcine-type STa (pSTa) or human-type STa (hSTa), uroguanylin, and guanylin peptides. The boxed sequence represents the toxicity domain, and heterogeneous amino acid residues are in light gray.

and 8th positions separate both guanylin peptides (referred to as guanylin and uroguanylin) from STa by forming only two disulfide bridges. The 6th (leucine) and 9th (asparagine) residues (equivalent to the 9th and 12th residues of STa) are shared by STa and uroguanylin but not guanylin (Fig. 1). Differences at these two residues likely dictate anti-STa antibody cross-reactivity with uroguanylin but not guanylin. Therefore, mutations at the 9th and/or the 12th residue of STa should enhance antigenic heterogeneity and divert STa further from uroguanylin in structure and potential antigenicity. By adding a mutation at the 14th residue of STa, which was demonstrated to play a role in the enterotoxicity and immunogenicity of STa, we generated three STa double mutants, $STa_{L9A/N12S}$, $STa_{N12S/A14T}$, and $STa_{L9A/A14H}$, and one triple mutant, $STa_{L9A/N12S/A14H}$. Genetically fusing three copies of each STa mutant to $mnLT_{R192G/L211A}$ produced four fusions: $3{\times}STa_{L9A/N12S}\text{-}mnLT_{R192G/L211A}$, $3{\times}STa_{N12S/A14T}\text{-}mnLT_{R192G/L211A}$, $3{\times}STa_{L9A/A14H}\text{-}mnLT_{R192G/L211A}$, and $3{\times}STa_{L9A/N12S/A14H}\text{-}mnLT_{R192G/L211A}$. Cloning each fusion gene (a single open reading frame [ORF]) at the NcoI and EagI sites of vector pET28α produced four recombinant strains to express fusion proteins without a 6$\times$His tag.

Four tagless double or triple mutant fusion proteins and tagless $3{\times}STa_{N12S}\text{-}mnLT_{R192G/L211A}$ (previously labeled $3{\times}STa_{N12S}\text{-}dmLT$) were verified with anti-cholera toxin (anti-CT) (Fig. 2) and anti-STa antisera. Expressed fusion proteins were confirmed by the lack of STa enterotoxicity. Two hundred micrograms of the fusion protein or 25 μl of the supernatant filtrates from a bacterial culture of each recombinant strain grown overnight did not significantly elevate intracellular cGMP levels in T-84 cells. When administered subcutaneously (s.c.), all fusion proteins were well tolerated by mice, as none of the mice showed adverse effects except for redness and mild swelling at the injection sites.

$STa_{toxoid}\text{-}mnLT_{R192G/L211A}$ fusion proteins induce anti-STa and anti-LT antibodies in s.c. immunized mice. All subcutaneously immunized mice developed anti-STa and anti-LT antibody responses (Fig. 3). Anti-STa IgG titers were 3.61 $\pm$ 0.46, 3.35 $\pm$ 0.65, 3.01 $\pm$ 0.61, and 3.53 $\pm$ 0.65 $\log_{10}$ units in serum samples of mice immunized with $3{\times}STa_{L9A/N12S}\text{-}mnLT_{R192G/L211A}$, $3{\times}STa_{N12S/A14T}\text{-}mnLT_{R192G/L211A}$, $3{\times}STa_{L9A/A14H}\text{-}$
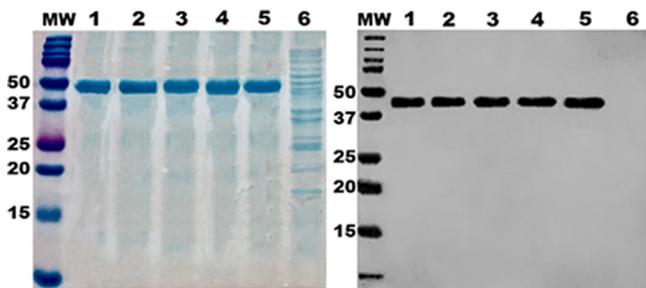
FIG 2 Expression and characterization of $3{\times}STa_{toxoid}\text{-}mnLT_{R192G/L211A}$ fusion proteins. (Left) SDS-PAGE with Coomassie blue staining to show the purity of purified fusion proteins. (Right) Western blotting for detection of fusion proteins with anti-CT rabbit antiserum (Sigma). Lanes: 1, $3{\times}STa_{L9A/N12S}\text{-}mnLT_{R192G/L211A}$; 2, $3{\times}STa_{N12S/A14T}\text{-}mnLT_{R192G/L211A}$; 3, $3{\times}STa_{L9A/N12S/A14H}\text{-}mnLT_{R192G/L211A}$; 4, $3{\times}STa_{L9A/A14H}\text{-}mnLT_{R192G/L211A}$; 5, $3{\times}STa_{N12S}\text{-}mnLT_{R192G/L211A}$; 6, total protein of host *E. coli* strain BL21; MW, molecular weight marker (in thousands).
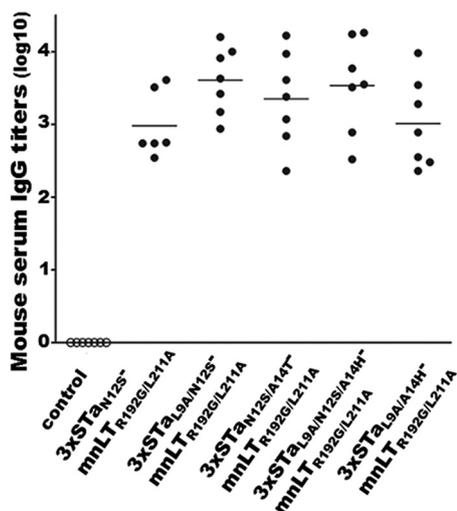
FIG 3 Anti-STa IgG titers (in $\log_{10}$ units) from serum samples of mice immunized s.c. with the $3\times STa_{N12S}$-$mnLT_{R192G/L211A}$, $3\times STa_{L9A/N12S}$-$mnLT_{R192G/L211A}$, $3\times STa_{N12S/A14T}$-$mnLT_{R192G/L211A}$, $3\times STa_{L9A/N12S/A14H}$-$mnLT_{R192G/L211A}$, or $3\times STa_{L9A/A14H}$-$mnLT_{R192G/L211A}$ fusion protein. Each dot represents the anti-STa IgG titer from a mouse in the immunized or control group. Bars indicate the mean IgG titers in each immunized group.

$mnLT_{R192G/L211A}$, and $3\times STa_{L9A/N12S/A14H}$-$mnLT_{R192G/L211A}$, respectively. Anti-LT IgG titers in these groups were 3.63 ± 0.20, 3.52 ± 0.24, 3.64 ± 0.21, and 3.62 ± 0.24 $\log_{10}$ units, respectively. Anti-STa and anti-LT IgG titers in the serum samples of mice immunized with $3\times STa_{N12S}$-$mnLT_{R192G/L211A}$ were 2.98 ± 0.46 and 3.85 ± 0.16 $\log_{10}$ units, respectively. No anti-STa or anti-LT IgG response was detected in serum samples of the control mice.

Serum samples of mice immunized with $3\times STa_{N12S}$-$mnLT_{R192G/L211A}$, $3\times STa_{L9A/N12S}$-$mnLT_{R192G/L211A}$, or $3\times STa_{N12S/A14T}$-$mnLT_{R192G/L211A}$ neutralize STa _in vitro_. Intracellular cGMP levels in T-84 cells incubated with STa and serum samples from mice immunized with $3\times STa_{N12S}$-$mnLT_{R192G/L211A}$, $3\times STa_{L9A/N12S}$-$mnLT_{R192G/L211A}$, or $3\times STa_{N12S/A14T}$-$mnLT_{R192G/L211A}$ were 1.87 ± 0.28, 1.82 ± 0.51, and 1.81 ± 0.41 pmol/ml, respectively. These levels were significantly lower than the cGMP levels in T-84 cells incubated with STa and serum samples of mice immunized with $3\times STa_{L9A/A14H}$-$mnLT_{R192G/L211A}$ (8.51 ± 0.58 pmol/ml; $P < 0.001$) or $3\times STa_{L9A/N12S/A14H}$-$mnLT_{R192G/L211A}$ (36.1 ± 2.35 pmol/ml; $P < 0.001$). The cGMP level in T-84 cells incubated with STa and control mouse serum samples was 46 ± 1.77 pmol/ml, and the cGMP level in cells incubated with STa alone was 50 ± 1.23 pmol/ml (Fig. 4).

Serum antibodies from mice immunized with the toxoid fusion $3\times STa_{N12S}$-$mnLT_{R192G/L211A}$ show little cross-reactivity with guanylin or uroguanylin in a competitive STa ELISA. Competitive STa enzyme-linked immunosorbent assays (ELISAs) showed that guanylin or uroguanylin (50 ng) exhibited little competition against the coated STa-ovalbumin conjugates for reacting with anti-STa antibodies in the sera of mice immunized with the toxoid fusion $3\times STa_{N12S}$-$mnLT_{R192G/L211A}$ (Fig. 5). Compared to the level of reactivity ($99.7\% \pm 0.4\%$) of anti-STa serum antibodies (at a 1:1,000 dilution) with the coated STa-ovalbumin conjugates (without competition from STa or guanylin peptides), the level of mouse serum anti-STa antibody reactivity with the STa-ovalbumin conjugates was determined to be $96.7\% \pm 2.2\%$ or $96.8\% \pm 2.5\%$ when 50 ng guanylin or 50 ng uroguanylin was added, respectively. This showed that only about 3% of the reactivity of anti-STa antibodies with STa-ovalbumin was reduced by guanylin or uroguanylin, while the background reactivity reading in wells without the STa-ovalbumin coating conjugate was $2.2\% \pm 0.4\%$. In contrast, when STa (50 ng) was included, the reactivity of anti-STa antibodies with the coated STa-ovalbumin conjugate was measured at $30.9\% \pm 2.3\%$, indicating that STa reacted with anti-STa
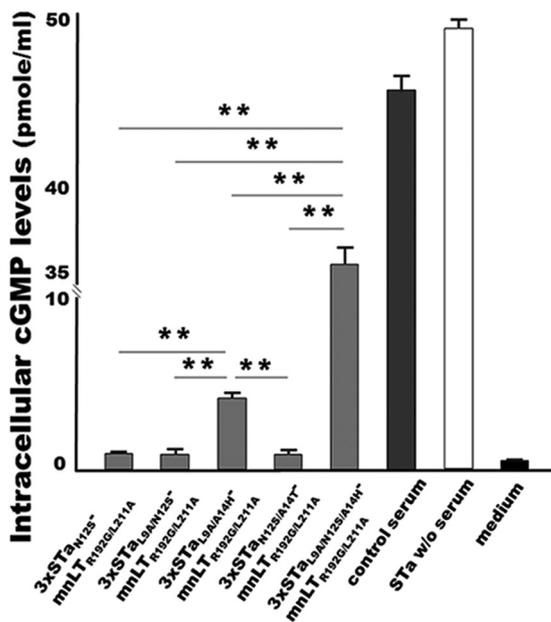
FIG 4 *In vitro* neutralization activity of mouse serum antibodies against STa. Intracellular cyclic GMP levels (picomoles per milliliter) in T-84 cells incubated with 2 ng STa and serum samples of mice immunized s.c. with the $3\times STa_{N12S}$-mnLT$_{R192G/L211A}$, $3\times STa_{L9A/N12S}$-mnLT$_{R192G/L211A}$, $3\times STa_{N12S/A14T}$-mnLT$_{R192G/L211A}$, $3\times STa_{L9A/N12S/A14H}$-mnLT$_{R192G/L211A}$, or $3\times STa_{L9A/A14H}$-mnLT$_{R192G/L211A}$ fusion protein were measured by using a cGMP EIA kit (Enzo Life). STa elevates intracellular cGMP levels in T-84 cells; neutralizing anti-STa antibodies prevent STa from stimulating cGMP production in T-84 cells. T-84 cells incubated with STa alone or STa with a control mouse serum sample were also included as controls. T-84 cells incubated with cell culture medium were used to show baseline cGMP levels in T-84 cells. ** indicates a *P* value of <0.01.

antibodies in mouse serum samples and blocked about 69% of the reactivity of anti-STa antibodies with coated STa-ovalbumin (Fig. 5).

Serum antibodies from mice immunized with the fusion $3\times STa_{L9A/N12S}$-mnLT$_{R192G/L211A}$, $3\times STa_{N12S/A14T}$-mnLT$_{R192G/L211A}$, $3\times STa_{L9A/A14H}$-mnLT$_{R192G/L211A}$, or $3\times STa_{L9A/N12S/A14H}$-mnLT$_{R192G/L211A}$ show little or limited cross-reactivity with guanylin or uroguanylin. Serum samples from mice immunized with $3\times STa_{L9A/N12S}$-mnLT$_{R192G/L211A}$, $3\times STa_{N12S/A14T}$-mnLT$_{R192G/L211A}$, or $3\times STa_{L9A/N12S/A14H}$-mnLT$_{R192G/L211A}$ showed little cross-reactivity with guanylin or uroguanylin, whereas serum samples of
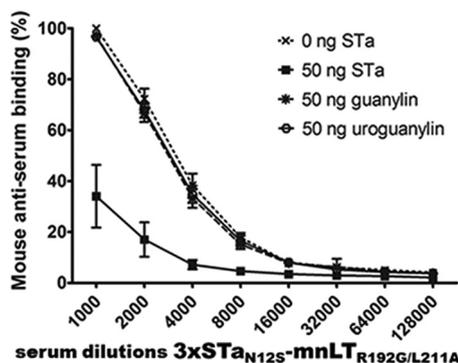
FIG 5 Competitive STa ELISAs to measure the cross-reactivity of anti-STa antibodies with guanylin and uroguanylin in serum samples of mice immunized s.c. with $3\times STa_{N12S}$-mnLT$_{R192G/L211A}$. ELISA plates coated with STa-ovalbumin conjugates (50 ng per well) were incubated with mouse serum samples (diluted 2-fold from 1:1,000 to 1:128,000) mixed with PBS, 50 ng STa, 50 ng guanylin, or 50 ng uroguanylin. The secondary antibody HRP-conjugated goat anti-mouse IgG (1:3,300; Sigma) and the TMB substrate (2C; KPL) were used to measure the optical absorbance at 405 nm. Reactivity is presented as a percentage, with the level of reactivity of mouse serum antibodies (at a 1:1,000 serum dilution) with the coated STa-ovalbumin conjugates being 100%.
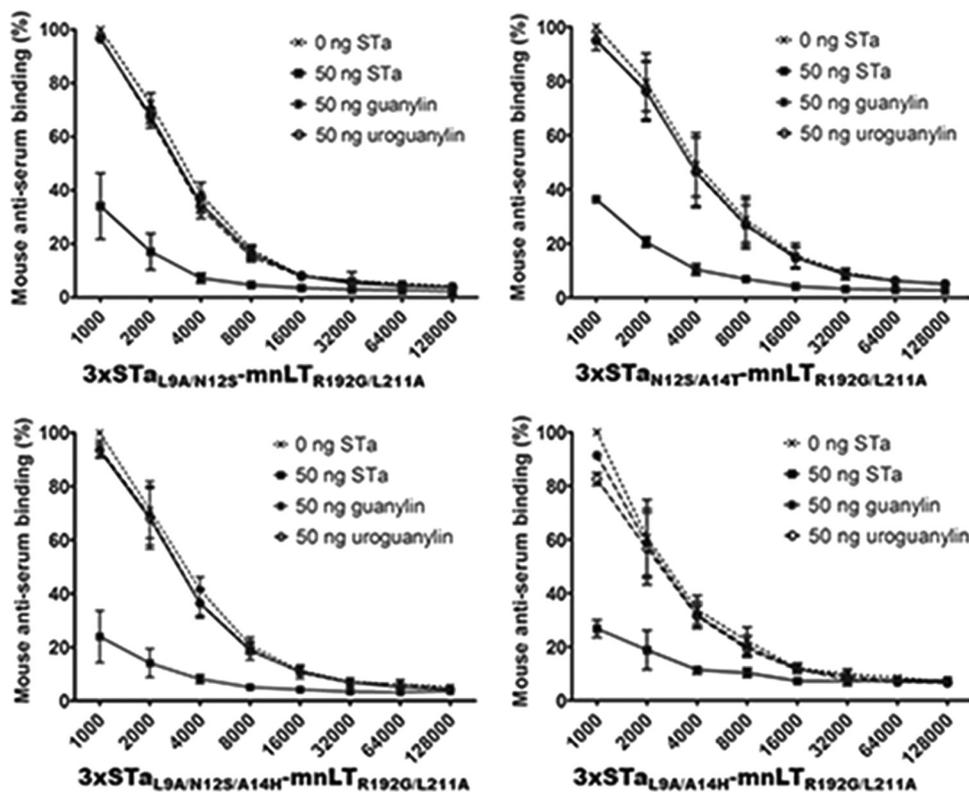
FIG 6 Competitive STa ELISAs to measure guanylin and uroguanylin cross-reactivity with anti-STa antibodies in serum samples of mice immunized s.c. with the $3\times STa_{L9A/N12S}$-mnLT$_{R192G/L211A}$, $3\times STa_{N12S/A14T}$-mnLT$_{R192G/L211A}$, $3\times STa_{L9A/N12S/A14H}$-mnLT$_{R192G/L211A}$, or $3\times STa_{L9A/A14H}$-mnLT$_{R192G/L211A}$ fusion protein. PBS, 50 ng STa toxin, 50 ng guanylin, or 50 ng uroguanylin mixed with serum dilutions from each immunization group was added to wells of competitive STa ELISA plates. The OD$_{405}$ was measured and converted to a percentage to indicate antibody reactivity, with the OD in the wells incubated with a 1:1,000 dilution of mouse serum and PBS having a value of 100% (0% antibody cross-reactivity).

mice immunized with $3\times STa_{L9A/A14H}$-mnLT$_{R192G/L211A}$ showed a low level of reactivity with guanylin peptides (Fig. 6). The levels of reactivity of coated STa-ovalbumin with serum samples (1:1,000 dilution) of mice immunized with $3\times STa_{L9A/N12S}$-mnLT$_{R192G/L211A}$, $3\times STa_{N12S/A14T}$-mnLT$_{R192G/L211A}$, and $3\times STa_{L9A/N12S/A14H}$-mnLT$_{R192G/L211A}$ were 96.3% ± 1.5%, 94.6% ± 3.0%, and 96.3% ± 4.2%, respectively, when guanylin was added. When uroguanylin was added, the levels of reactivity were 95.8% ± 1.4%, 95.8% ± 1.4%, and 95.8% ± 4.3%, respectively. The background reactivity readings ranged from 2.2% ± 0.2% to 3.6% ± 0.2%.

The levels of reactivity of the coated STa-ovalbumin conjugates with serum samples (1:1,000 dilution) of mice immunized with $3\times STa_{L9A/A14H}$-mnLT$_{R192G/L211A}$ were 90.1% ± 3.3% and 81.3% ± 2.6% when guanylin and uroguanylin were included, respectively. This showed that guanylin and uroguanylin cross-reacted with 10% and 19% of anti-STa antibodies or blocked 10% and 19% of the anti-STa antibody reactivity with the coated STa-ovalbumin conjugates, respectively.

Comparatively, when 50 ng STa was included, the levels of reactivity of the coated STa-ovalbumin conjugates with serum antibodies of mice immunized with $3\times STa_{L9A/N12S}$-mnLT$_{R192G/L211A}$, $3\times STa_{N12S/A14T}$-mnLT$_{R192G/L211A}$, $3\times STa_{L9A/N12S/A14H}$-mnLT$_{R192G/L211A}$, and $3\times STa_{L9A/A14H}$-mnLT$_{R192G/L211A}$ were 33.8% ± 11.3%, 36% ± 2.2%, 33.8% ± 8.8%, and 26.4% ± 2.5%, respectively (Fig. 6).

DISCUSSION

The cross-reactivity of anti-STa antibody with guanylin peptides (guanylin and uroguanylin) has not been well investigated. It was reported previously that STa did not cross-react with an antiguanylin monoclonal antibody (MAb) (21). A recent study

examined two polyclonal antibodies, one against STa type Ib (hSTa [human-type STa]) and the other against STa type Ia (pSTa [porcine-type STa]), as well as four monoclonal antibodies (one against hSTa and three against pSTa) and found that rabbit anti-STa antiserum did not cross-react with guanylin but cross-reacted with uroguanylin at a low level (14). Those findings suggested a possible solution for the design of ETEC vaccines for anti-STa antibodies that do not cross-react with guanylin and uroguanylin. Data from the present study showed that the constructed fusion proteins carrying an STa double or triple mutant induced anti-STa and anti-LT antibody responses as effectively as did the STa single mutant fusion $3\times STa_{N12S}$-$mnLT_{R192G/L211A}$ in subcutaneously immunized mice, and $3\times STa_{L9A/N12S}$-$mnLT_{R192G/L211A}$ and $3\times STa_{N12S/A14T}$-$mnLT_{R192G/L211A}$ induced antibodies that neutralized STa enterotoxicity without cross-reacting with guanylin and uroguanylin.

The introduction of double or triple mutations into STa significantly reduced the homology of STa with guanylin and uroguanylin. These STa mutants are preferred as ETEC vaccine antigens to induce antibodies that neutralize STa enterotoxicity but do not cross-react with guanylin peptides. Primary sequence comparisons indicated that while both guanylin peptides differ from STa at the 3rd, 8th, and 10th residues, guanylin further differs from STa (and uroguanylin) at the 6th and 9th residues (equal to the 9th and 12th residues of STa) (Fig. 1). The heterogeneity at these two residues (the 6th and 9th residues) separates guanylin further from STa and may directly result in a lack of anti-STa antibody cross-reactivity with guanylin (14). In contrast, sharing of the 6th and 9th residues by uroguanylin and STa likely causes anti-STa antibody cross-reactivity with uroguanylin. Data from this study showed that antibodies from fusions carrying STa mutated at these two residues had little cross-reactivity with guanylin or uroguanylin. The data also suggested that the heterogeneity at the 9th residue of uroguanylin (equal to the 12th residue of STa) likely plays a more important role in diverting STa mutants from guanylin peptides antigenically, since anti-STa antibodies derived from fusions carrying STa peptides mutated at the 12th asparagine residue (alone or combined with other mutations) showed no or little cross-reactivity with guanylin and uroguanylin. In contrast, serum antibodies from mice immunized with $3\times STa_{L9A/A14H}$-$mnLT_{R192G/L211A}$, which had the native 12th residue of STa, had 10% and 19% cross-reactivities with guanylin and uroguanylin, respectively. Future studies to compare the structural or antigenic divergence of $STa_{L9A/A14H}$ with those of the other four mutants may help us to understand differences in anti-STa antibody cross-reactivity with guanylin and uroguanylin.

Five STa mutants with mutations at the 9th, 12th, and 14th residues were targeted for the construction of toxoid fusions and for the examination of antibody cross-reactivity with guanylin peptides in this study. Selecting the 9th and 12th residues is obvious because STa shares the 9th residue (leucine) and the 12th residue (asparagine) with uroguanylin but not with guanylin. Additionally, the 9th residue (leucine) was thought to be associated with STa antigenic properties together with enterotoxicity. STa lost its reactivity with anti-STa antibodies and also enterotoxicity when the leucine residue at the 9th position was replaced with glutamine, arginine, glycine, or serine. On the other hand, STa mutants retained some anti-STa antibody reactivity and also some enterotoxicity when the leucine residue at the 9th position was replaced with isoleucine or lysine (22). Later antigenicity and enterotoxicity studies confirmed that a majority of STa mutants with mutations at the 9th residue had 90% of the antigenicity and enterotoxicity of STa reduced, and only a few mutants, including STa_{L9M}, STa_{L9V}, and STa_{L9F}, retained a portion of the antigenicity and also the enterotoxicity of STa, although a few exceptional mutants, including STa_{L9G}, STa_{L9S}, and STa_{L9A}, retained some STa antigenicity but not STa enterotoxicity (16). The STa_{L9A} mutant was selected because the toxoid fusion $3\times STa_{L9A}$-$mnLT_{R192G/L211A}$ ($3xSTa_{L9A}$-$dmLT$) was previously demonstrated to induce neutralizing anti-STa IgG and IgA antibodies (10). The results from the present study also confirmed that a single mutation of the asparagine residue at the 12th position to serine or a double mutation of the 12th and 9th residues (leucine to alanine) did not significantly alter the propensity of STa for antigenicity, which was

demonstrated by the induction of neutralizing anti-STa antibodies by $3 \times STa_{N12S}$-$mnLT_{R192G/L211A}$ and $3 \times STa_{L9A/N12S}$-$mnLT_{R192G/L211A}$. These results indicated that mutations at the 12th residue or at both the 9th and 12th residues separated STa further from guanylin and uroguanylin, which was proven by the lack of cross-reactivity with guanylin or uroguanylin from antibodies derived from $3 \times STa_{N12S}$-$mnLT_{R192G/L211A}$, $3 \times STa_{L9A/N12S}$-$mnLT_{R192G/L211A}$, or $3 \times STa_{L9A/N12S/A14H}$-$mnLT_{R192G/L211A}$. Mutation of the 14th residue of STa was also included in this study even though this alanine residue is identical among STa and the guanylin peptides. The alanine residue at the 14th position was previously suggested to play an important role in the enterotoxicity and antigenicity of STa (10, 16, 23). Additionally, the STa_{N12S} single mutant was used as a reference mutant. The STa single mutant fusion $3 \times STa_{N12S}$-$mnLT_{R192G/L211A}$ induced antibodies that neutralized STa *in vitro*, protected piglets against STa^+ ETEC diarrhea *in vivo* (10–12), and is currently considered the leading toxin antigen for ETEC vaccines. Data from this study suggested that the single mutant fusion $3 \times STa_{N12S}$-$mnLT_{R192G/L211A}$ was equally as effective as the double mutant fusions at inducing neutralizing and non-cross-reacting anti-STa antibodies.

The competitive STa ELISA was optimized by using 50 ng STa-ovalbumin coating antigen, 50 ng competitive antigen (guanylin, uroguanylin, or STa), and serum dilutions from 1:1,000 to 1:128,000 to examine anti-STa antibody cross-reactivity. Checkerboard tests showed that excessive anti-STa antibodies or insufficient competing agents (guanylin or uroguanylin) skewed anti-STa antibody reactivity with the coating antigen STa-ovalbumin, masking the detection of any cross-reactivity with guanylin or uroguanylin. On the other hand, a lower dose of coating antigen or more-diluted mouse serum samples resulted in low antibody reactivity to the coating antigen, as indicated by lower optical density at 405 nm (OD_{405}) readings. It needs to be pointed out that the cross-reactivity of guanylin and uroguanylin currently measured in competitive ELISAs is reported relative to the reactivity of anti-STa antibodies with the coating antigen without any competitive molecules. Additionally, future studies using a pig challenge model or a controlled human challenge model (CHIM) to examine anti-STa and anti-LT antibodies induced by these toxoid fusions against ETEC diarrhea will help us to better assess their candidacy for ETEC vaccine development.

In conclusion, this study showed that the STa single, double, and triple mutants STa_{N12S}, $STa_{L9A/N12S}$, $STa_{N12S/A14T}$, $STa_{L9A/A14H}$, and $STa_{L9A/N12S/A14H}$, after being genetically fused to a monomeric LT mutant ($mnLT_{R192G/L211A}$), induced antibody responses to STa and LT. Antibodies derived from the fusions $3 \times STa_{N12S}$-$mnLT_{R192G/L211A}$, $3 \times STa_{L9A/N12S}$-$mnLT_{R192G/L211A}$, and $3 \times STa_{N12S/A14T}$-$mnLT_{R192G/L211A}$ neutralized STa *in vitro* and also did not cross-react with guanylin or uroguanylin. These results indicate that the toxoid fusions $3 \times STa_{N12S}$-$mnLT_{R192G/L211A}$, $3 \times STa_{L9A/N12S}$-$mnLT_{R192G/L211A}$, and $3 \times STa_{N12S/A14T}$-$mnLT_{R192G/L211A}$ are potentially desirable antigens for developing safe ETEC vaccines.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* bacteria and plasmids used in this study are included in Table 1. *E. coli* recombinant strain 9471, which was modified from 9331 (10) by removing the 6-histidine tag to express the tagless $3 \times STa_{N12S}$-$mnLT_{R192G/L211A}$ toxoid fusion, was used as the DNA template for toxoid fusion construction. Vector pET28α (Novagen, Madison, WI) was used to clone toxoid fusion genes, and *E. coli* strain BL21 was used to express toxoid fusion proteins.

Construction of the $3 \times STa_{toxoid}$-$mnLT_{R192G/L211A}$ toxoid fusion. To avoid confusion with the holotoxin-structured double mutant $LT_{R192G/L211A}$ (dmLT), we designated the monomeric LT mutant $mnLT_{R192G/L211A}$. This mutant consists of one LT_A subunit and one LT_B subunit as a monomer peptide (from a single ORF), with the A subunit mutated at positions 192 and 211. Splicing by overlap extension (SOE) PCR was used to mutate the STa gene (*astA*) for double or triple STa mutants and to fuse three copies of each STa mutant to the $mnLT_{R192G/L211A}$ gene for the construction of $3 \times STa_{toxoid}$-$mnLT_{R192G/L211A}$ fusion genes by using the PCR primers listed in Table 2. As described previously (9, 10), six nucleotide fragments were initially PCR amplified with *Pfu* DNA polymerase (Agilent Technologies, Santa Clara, CA) and primer pairs T7-F/STa_{toxoid}-R, STa_{toxoid}-F/LT_{A310}-R, LT_{A310}-F/STa_{toxoid}-R, STa_{toxoid}-F/LT_{B44}-R, LT_{B44}-F/STa_{toxoid}-R, and STa_{toxoid}-F/T7-R. Primers STa_{toxoid}-F and STa_{toxoid}-R were specific for each STa double or triple mutant. Overlapping the first two fragments, the third and the fourth fragments, and the last two amplicons generated three fragments carrying the first, second, and third copies of an STa toxoid and a part of $mnLT_{R192G/L211A}$, respectively. Overlapping these three fragments together in a single

TABLE 1 *E. coli* strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
BL21	F^- *ompT hsdS*$(r_B^-$ $m_B^-)$ *gal dcm*	GE Healthcare
9471	$3\times STa_{N12S}$-$mnLT_{R192G/L211A}$ recombinant strain	This study
9600	$3\times STa_{L9A/N12S}$-$mnLT_{R192G/L211A}$ recombinant strain	This study
9601	$3\times STa_{N12S/A14T}$-$mnLT_{R192G/L211A}$ recombinant strain	This study
9602	$3\times STa_{L9A/N12S/A14H}$-$mnLT_{R192G/L211A}$ recombinant strain	This study
9603	$3\times STa_{L9A/A14H}$-$mnLT_{R192G/L211A}$ recombinant strain	This study
Plasmids		
pET28α		Novagen
p9463	Tagless $3\times STa_{N12S}$-$mnLT_{R192G/L211A}$ gene in pET28α	This study
p9596	Tagless $3\times STa_{L9A/N12S}$-$mnLT_{R192G/L211A}$ gene in pET28α	This study
p9597	Tagless $3\times STa_{N12S/A14T}$-$mnLT_{R192G/L211A}$ gene in pET28α	This study
p9598	Tagless $3\times STa_{L9A/N12S/A14H}$-$mnLT_{R192G/L211A}$ gene in pET28α	This study
p9599	Tagless $3\times STa_{L9A/A14H}$-$mnLT_{R192G/L211A}$ gene in pET28α	This study

SOE PCR yielded a $3\times STa_{toxoid}$-$mnLT_{R192G/L211A}$ toxoid fusion gene. Each toxoid fusion gene was digested with NcoI and EagI and ligated into the pET28a vector.

Expression and characterization of the $3\times STa_{toxoid}$-$mnLT_{R192G/L211A}$ fusion protein. Toxoid fusion proteins without a $6\times$His tag expressed in *E. coli* strain BL21 were extracted and refolded as described previously (10, 24). Briefly, recombinant bacteria of each toxoid fusion (from a single colony) grown overnight in 5 ml lysogeny broth (LB) were added to 200 ml $2\times$YT medium ($2\times$ yeast extract, Tryptone) and continuously cultured until the OD reached 0.6. Bacteria were then induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG; Sigma, St. Louis, MO). Bacteria were collected and processed for the extraction of inclusion body proteins with bacterial protein extraction reagent (B-PER; Pierce, Rockford, IL) according to the manufacturer's protocol. Extracted inclusion body proteins were solubilized and refolded by using a protein refolding kit (Novagen) according to the manufacturer's protocol.

Refolded toxoid fusion proteins were electrophoresed in a 12% sodium dodecyl sulfate-polyacrylamide gel, visualized by Coomassie blue staining, and characterized by Western blotting with rabbit anti-CT antiserum (Sigma) and rabbit anti-STa antiserum (provided by D. C. Robertson at Kansas State University), as previously described (10).

Subcutaneous immunization of mice with the $3\times STa_{toxoid}$-$mnLT_{R192G/L211A}$ fusion protein. Groups of six or seven adult female BALB/c mice were subcutaneously immunized with 40 μg of each refolded toxoid fusion protein. Two micrograms of holotoxin-structured dmLT (provided by the Walter Reed Army Institute of Research, Silver Spring, MD) was included in each immunization as an adjuvant. A group of seven mice immunized with protein buffer was used as the control. Each mouse received two boosters at intervals of 2 weeks. All mice were sacrificed 2 weeks after the second booster. The mouse study was performed according to 1996 National Research Council guidance (25) and was approved and supervised by the Kansas State University IACUC.

Mouse serum anti-STa and anti-LT IgG antibody titration. Serum samples from each immunized or control mouse were titrated for anti-STa and anti-LT IgG antibodies as previously described (7, 8, 10, 11). Briefly, 10 ng STa-ovalbumin conjugates (provided by D. C. Robertson at Kansas State University) was used to coat each well of Costar plates (Corning Inc., Corning, NY) to titrate anti-STa antibodies, and

TABLE 2 PCR primers used to mutate the STa gene (*estA*) and to construct $3\times STa_{-toxoid}$-$mnLT_{R192G/L211A}$ toxoid fusion genes in this study

Primer	Nucleotide sequence (5'→3')
T7-F	TAATACGACTCACTATAGGG
T7-R	TGCTAGTTATTGGTCAGGGGT
LT_{B44}-F	ATCATTACATTTAAGAGCGGCGC
LT_{B44}-R	GCGCCGCTCTTAAATGTAATGAT
LT_{A310}-F	TATGTTATAGCGACAGCACC
LT_{A310}-R	GGTGCTGTCGCTATAACATA
$STa_{L9A/N12S}$-F	AGCAATTACTGCTGTGAAGCATGTTGTAGCCCTGCTTGTACCGGGTGC
$STa_{L9A/N12S}$-R	GCACCCGGTACAAGCAGGGCTACAACATGCTTCACAGCAGTAATTGCT
$STa_{L9A/A14H}$-F	AATTACTGCTGTGAAGCATGTTGTAATCCTCACTGTACCGGGTGCTAT
$STa_{L9A/A14H}$-R	ATAGCACCCGGTACAGTGAGGATTACAACATGCTTCACAGCAGTAATT
$STa_{N12S/A14T}$-F	AATTACTGCTGTGAATTGTGTTGTAGCCCTACGTGTACCGGGTGCTAT
$STa_{N12S/A14T}$-R	ATAGCACCCGGTACACGTAGGGCTACAACACAATTCACAGCAGTAATT
$STa_{L9A/N12S/A14H}$-F	AATTACTGCTGTGAAGCATGTTGTAGCCCTCACTGTACCGGGTGCTAT
$STa_{L9A/N12S/A14H}$-R	ATAGCACCCGGTACAGTGAGGGCTACAACATGCTTCACAGCAGTAATT

each well of 2HB plates (Thermo Scientific, Rochester, NY) was coated with 100 ng LT (List Biological Laboratories Inc., Campbell, CA) to titrate anti-LT antibodies. Mouse serum samples were diluted 2-fold from 1:200 to 1:256,000. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:3,300; Sigma) was used as the secondary antibody. The tetramethylbenzidine (TMB) microwell peroxidase substrate kit (KPL, Gaithersburg, MD) was used to measure the OD_{405}.

Mouse serum antibody neutralization against STa. Mouse serum samples from each immunization group and the control group were examined for neutralization activity against STa by using T-84 cells and a cGMP enzyme immunoassay (EIA) kit (Enzo Life, Farmingdale, NY) as previously described (7, 8, 10). Briefly, a 30-μl serum sample mixed with 2 ng STa (in 150 μl Dulbecco's modified Eagle's medium [DMEM]) was incubated at room temperature for 1 h. The mixture was added to T-84 cells (in Eagle's minimum essential medium, with a final volume of 1 ml). After 1 h of incubation in a CO_2 incubator, T-84 cells were lysed, and intracellular cGMP concentrations (picomoles per milliliter) in T-84 cells were measured with the cGMP EIA kit according to the manufacturer's protocol (Enzo Life).

Mouse serum antibody cross-reactivity with guanylin and uroguanylin. A competitive STa ELISA (26, 27) was used to measure guanylin or uroguanylin cross-reactivity with anti-STa antibodies derived from each toxoid fusion. Briefly, Costar plates (Corning) coated with STa-ovalbumin conjugates (50 ng per well) were incubated with 2-fold dilutions of mouse serum (from 1:1,000 to 1:128,000) from each immunization group and phosphate-buffered saline (PBS), 50 ng STa toxin, 50 ng guanylin, or 50 ng uroguanylin. Wells without the STa-ovalbumin conjugate coating antigen were included to measure background readings. After incubation for 1 h at 37°C, plates were washed and then incubated with HRP-conjugated goat anti-mouse IgG (1:3,300; Sigma), followed by TMB peroxidase substrates (KPL). The OD_{405} was then measured.

Data analyses. Mouse serum samples were examined in duplicates. Antibody titration and neutralization assays were replicated two times, and antibody cross-reactivity assays were repeated three times. Differences between treatment groups were analyzed with two-sided nonparametric Mood's median test by using Sigma XL (Kitchener, ON, Canada) with 95% confidence.

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