Microbiology and Immunology

Microbiol Immunol 2017; 61: 272–279
doi: 10.1111/1348-0421.12491

ORIGINAL ARTICLE

A Chimeric protein of CFA/I, CS6 subunits and LTB/STa toxoid protects immunized mice against enterotoxigenic *Escherichia coli*

Narges Zeinalzadeh[1], Ali Hatef Salmanian[2], Goli Goujani[2], Jafar Amani[3], Ghasem Ahangari[4], Asal Akhavian[2] and Mahyat Jafari[2]

[1]Department of Animal Science, Faculty of Natural Science, University of Tabriz, Tabriz, [2]Department of Agricultural Biotechnology, National Institute of Genetic Engineering and Biotechnology, Shahrak-e- Pajoohesh, km 15, Tehran-Karaj Highway, [3]Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences and [4]Department of Medical Biotechnology, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran

ABSTRACT

Enterotoxigenic *Escherichia Coli* (ETEC) strains are the commonest bacteria causing diarrhea in children in developing countries and travelers to these areas. Colonization factors (CFs) and enterotoxins are the main virulence determinants in ETEC pathogenesis. Heterogeneity of CFs is commonly considered the bottleneck to developing an effective vaccine. It is believed that broad spectrum protection against ETEC would be achieved by induced anti-CF and anti-enterotoxin immunity simultaneously. Here, a fusion antigen strategy was used to construct a quadrivalent recombinant protein called 3CL and composed of CfaB, a structural subunit of CFA/I, and CS6 structural subunits, LTB and STa toxoid of ETEC. Its anti-CF and antitoxin immunogenicity was then assessed. To achieve high-level expression, the 3CL gene was synthesized using *E. coli* codon bias. Female BALB/C mice were immunized with purified recombinant 3CL. Immunized mice developed antibodies that were capable of detecting each recombinant subunit in addition to native CS6 protein and also protected the mice against ETEC challenge. Moreover, sera from immunized mice also neutralized STa toxin in a suckling mouse assay. These results indicate that 3CL can induce anti-CF and neutralizing antitoxin antibodies along with introducing CFA/I as a platform for epitope insertion.

Key words challenge, colonization factors, enterotoxigenic *E. coli*, STa toxoid.

Enterotoxigenic ETEC, the commonest cause of non-inflammatory diarrhea in developing countries, is responsible for approximately 20% of all diarrheal episodes in children in these areas (1). It is also known as one of the main cause of diarrhea in travelers to high risk areas and is accordingly known as travelers' diarrhea (2). Enterotoxins and CFs are major virulence factors in ETEC infections (3). ETEC uses colonization factors to attach to the epithelial surface of the small intestine, a vital early step in diarrhea pathogenesis, and then produces enterotoxins that act on the epithelial cells.

Enterotoxins including LT and STa, disrupt intestinal fluid homeostasis and cause hyper-secretion of fluid and electrolytes through activation of adenyl cyclase by LT or guanylate cyclase by STa in intestinal epithelial cells (3).

No effective vaccine is currently available for protecting humans and animals against ETEC

Correspondence
Ali Hatef Salmanian, Department of Agricultural Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Shahrak-e- Pajoohesh, km 15, Tehran-Karaj Highway, Tehran, Iran, P.O. Box 14965-161, Tehran, Iran. Tel: +98 21 4458 0365; fax: +98 21 4478 7365; email: salman@nigeb.ac.ir

Received 24 September 2016; revised 2 May 2017; accepted 18 May 2017.

List of Abbreviations: CF, colonization factor; ETEC, enterotoxigenic *Escherichia Coli*; i.p., intraperitoneal; LT, heat-labile; LTB, nontoxic B subunit of LT; MU, mouse unit; ST, heat stable; STa, heat stable toxin type Ib.

infections (4). The heterogeneity of ETEC colonization factors and potent toxicity of enterotoxins are the critical obstacles to developing effective vaccines against ETEC (5, 6). Given that more than 25 different and immunologically heterogeneous CFs have been characterized among ETEC strains (5), vaccines that target only one of these CFs can provide protection only against ETEC that expresses the same or homologous CFs.

The enterotoxicity potential of LT and STa decreases their opportunity to be considered as antigens for developing safe vaccines. Moreover, STa, a 19-amino-acid peptide, is a small and poorly immunogenic molecule and thus unsuitable for use as a vaccine target (6, 7). In the case of LT, several detoxified derivatives have been introduced and used as vaccine candidates (8–10). LTB, which has high antigenicity and adjuvanticity potentials, has been used not only in ETEC vaccines, but also in formulating other vaccines (11, 12). Moreover, because over two-thirds of human ETEC strains are STa^+ (13) and STa and LT have no epitopes in common, and because anti-LT immunity does not confer any cross-protection against STa toxin, STa antigens must be considered when attempting to design broadly effective vaccines against ETEC diarrhea (14, 15). By removal or reducing of STa toxicity and increasing its immunogenicity, this small molecule could be used as a safe component in multimeric vaccines. (15). Many studies have performed genetic fusion or chemical conjugation of STa peptides to an immunogenic carrier protein such as OmpC (16) or LT derivatives (10, 14, 17) to produce vaccines for inducing anti-STa immunity and demonstrated that the resultant fusion molecules are safe and can stimulate neutralizing antibodies against both STa and the carrier molecules. In this regard, several studies have also applied the CFs structural subunits as carrier molecules to develop anti-CF and anti-STa immunity (18, 19). In fact, anti-toxin and anti-adhesin immunity can be simultaneously stimulated by adhesin–toxoid chimeric antigens (10, 14). Recently, we designed a chimeric construct containing CfaB (CFA/I major subunit of ETEC fimbriae), CssA and CssB proteins (structural subunits of CS6) and the antigenic subunit of LT toxin, LTB, as a candidate vaccine against ETEC, designated as 3CL (20). In this construct, STa toxoid molecule is exposed as an epitope in the CfaB structure. Zhang *et al.* have reported this epitope as "a shorter peptide of porcine STa toxoid STa13"; its toxicity is reduced and its anti-STa immunogenicity increased when it is fused to a LT toxoid protein. In this STa derivative, alanine is substituted for the 13th amino acid, glutamine (19).

The bioinformatics features of this adhesin–toxoid chimeric antigen have been reported, as have the results of assessing its expression, purification and characterization by using immunoblot methods (20). In a subsequent step, in the current study the purified fusion protein was used to immunize mice to examine its anti-toxoid and anti-CFs antigenicity and ability to produce neutralizing antibodies. To evaluate the protection potential of induced immunity, the immunized mice were challenged with a LT, STa, CFA/I-producing ETEC strain.

MATERIAL AND METHODS

Bacterial strains, cell lines and media

E. coli DH5α and BL21 (DE3) (Pasteur Institute of Iran, Tehran, Iran) were used for cloning and expression of chimeric protein, respectively. ETEC strain CFA/I^+, LT^+, STa^+ was obtained from the reference laboratory of Buali hospital (Tehran, Iran). Purified CS6 (kindly provided by Dr. Nabendu S. Chatterjee, National Institute of Cholera and Enteric Diseases, Kolkata, India) was used for titration of anti-CS6 antibody. ETEC strains were grown in CF medium containing 1% casamino acids, 0.15% yeast extract, 0.005% $MgSO_4.7H_2O$ and 0.0005% $MnCl_2.2H_2O$, pH 7.4. For CF solid medium, 2% agar was added. The *E. coli* strains were cultured in LB medium supplemented with kanamycin (100 μg/mL).

Design and construction of chimeric 3CL

CfaB, CFA/I major subunit, CssA/B, CS6 subunits and LTB were selected for designing a chimeric construct. STa toxoid peptide (CCELCCNPQCTGCY) was inserted as a foreign epitope into the CfaB sequence. Bioinformatics analyses were used to select the best position for insertion of the STa toxoid and the best order of subunits in the fusion protein, as reported previously (20). Three and four repeats of EAAAK were used as linkers to generate a multivalent chimeric protein. The concluding chimeric gene (GeneBank accession number: JX481981.1) was then synthesized and cloned into pET28a.

Molecular cloning, expression, purification and characterization of 3CL chimeric protein

Recombinant pET28+*3cl* vector sub-cloned into *E. coli* BL21 (DE3) and recombinant 3CL protein was expressed by isopropyl b-D-1-thiogalactopyranoside induction. The fusion protein was purified by His-tagged sites at amino and carboxyl terminal sites and characterized by commercial anti-LTB mAb and anti-CfaB, anti-CssA and anti-CssB sera, which were produced in our

laboratory by immunizing mice with each of the recombinant proteins. Western blot analysis was also used to characterize the chimeric protein, as described previously (20). In this analysis native CS6 antiserum was used as the primary antibody. Moreover, the anti-chimeric protein serum was used as the primary antibody for detecting each of the subunit proteins and also for purifying native CS6 protein by western blotting.

Immunization of mice with purified fusion protein and antibody titration

Female BALB/C mice (6–8 weeks old) (Pasteur Institute of Iran) were used as the animal model. The animals were immunized s.c. with 10 μg purified protein in an equal volume of Freund's complete adjuvant (Sigma–Aldrich, St Louis, MO, USA). Two extra doses using Freund's incomplete adjuvant were injected at biweekly intervals as booster doses. Seven days before challenge, the animals were injected with 5 μg protein i.p. as an additional booster dose (fourth dose in total). For a control group, mice were injected with PBS instead of chimeric protein.

Blood and fecal samples were collected before each injection and the samples stored at −70°C until use. ELISA was used to titrate anti-3CL antibodies in serum samples, as described previously (21). 3CL protein (5 μg) was coated as antigen in the wells of ELISA plates (Nunc, Roskilde, Denmark), immunized mice sera were used as primary antibodies (diluted 1:200 in PBST) and HRP-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-mouse IgA were used as secondary antibodies (diluted 1:40,000 and 1:10,000, respectively, in PBST). The OD was measured at 492 nm on a microplate reader (Bio-Rad, Hercules, CA, USA).

To titrate anti-3CL secretory IgA antibody, fecal samples were collected daily from each mouse and frozen at −70°C until use. For ELISA, 1 g of the collected fecal samples was mixed thoroughly with 500 μL of PBS and centrifuged (10 min, 6000 rpm, 4°C). The supernatants were serially diluted in PBST and added to the ELISA plates, which were then incubated for 1 hr at 37°C. The plates were washed three times in PBST and HRP goat anti-mouse IgA antibodies (1:10,000 in PBST) (Sigma–Aldrich) and added to the ELISA plates, which were then incubated for 30 min at 37°C and washed three times in PBST. Citrate buffer (100 μL) containing 0.06% (w/v) of o-phenylenediaminedihydrochloride (Sigma–Aldrich) and 0.06% (v/v) hydrogen peroxide were added to the wells at room temperature for 15 min. The reactions were stopped with 100 μL of 2 M H_2SO_4 and the OD_{492} read.

Suckling mouse assay for STa neutralization activity of anti-3CL serum

The potential of the antibodies from immunized mice to neutralize STa toxicity was determined using an *in vivo* neutralization assay in a suckling mouse model (22). Briefly, one MU of enterotoxicity of crude culture of an ETEC STa^+ strain was delineated as the minimum volume needed to cause a positive response, that is, G/C (weight ratio of Gut to the remaining Carcass) ratios of >0.090. ETEC (STa^+) strain was grown overnight in CF medium at 37°C and 180 rpm. This culture was used to inoculate 50 mL of fresh CF medium. The culture was grown for 18 hr under 200 rpm shaking at 37°C and then centrifuged at 5000 rpm for 20 min. The supernatant was used as a STa crude source for enterotoxin. Three-day-old newborn suckling mice were separated from their mothers just before the test. Crude toxin (1 MU) was pretreated with 1:0.5 and 1:1 ratios of 3CL antiserum for 1 hr at 37°C and then inoculated intragastrically into newborn mice. The inoculated mice were kept at 25°C for 3 hr and then killed with chloroform. The abdomen was opened, the small intestines removed and weighed together and the G/C ratio calculated. Ratios of less than 0.090 were considered negative whereas those over 0.090 were considered positive.

Anti-CS6 antibody titration

To evaluate the neutralizing activity of anti-3CL serum against purified CS6, 100 ng of CS6 was coated in each well of an ELISA plate. Anti-3CL serum was used as the primary antibody (diluted 1:200 in PBST) and HRP conjugated goat anti-mouse IgG as the secondary antibody (diluted 1: 40,000 in PBST).

ETEC challenge of immunized mice

The immunized mice were challenged 6 months after the first immunization. For i.p challenge, 1×10^7 CFU of wild type ETEC was diluted in 100 μL of sterile PBS and injected. The challenged animals were followed for evidence of disease and death for 7 days.

Statistical analysis

The data are presented as mean ± SD. A one-way anova was used to compare the means between groups of more than two. SPSS 21.0 software was used for performing statistical analyses. Value of $P < 0.05$ were considered to denote statistically significant differences and $P < 0.01$ clearly significant differences.

RESULTS

Characterization of chimeric protein by western blot analysis

Purified 3CL chimeric protein was characterized by detection with anti-rCfaB, anti-rCssA, anti-rCssB and anti-CS6 sera and anti-LTB antibody (5). This characterization was confirmed by detection of recombinant CfaB, CssA, CssB and LTB with anti-3CL serum in western blotting (Fig. 1a,b). Because no $CS6^+$ ETEC strain is available, an ELISA (data not shown) and western blotting assay was done against purified CS6 protein to evaluate the neutralization activity of anti-3CL serum (Fig. 1b), both of which confirmed the specificity of anti-3CL serum for native purified CS6.

Antibody responses of immunized animals to chimeric protein

The immunized mice showed a significant serum IgG response to purified 3CL protein (Fig. 2a), however, IgAs response in sera and fecal samples did not differ significantly from controls (Fig. 2b). As Figure 2a shows, following boosting immunization, robust antibody responses were stimulated with a peak on Day 7.

Neutralization of STa enterotoxin

Because of the poor antigenicity of STa, induction of neutralizing antibodies against it is difficult. Therefore an *in vivo* suckling mouse model in which the antisera of immunized animals were able to neutralize STa toxin was used to determine the neutralizing capacity of antibodies induced against STa epitope of the recombinant chimeric protein. The response in the suckling mouse assay using one MU of crude culture (G/C <0.09) was found to be negative (Fig. 3), indicates that treating 1 MU (40 μL) of crude culture with the same ratio (1:1) of sera can neutralize the toxin, which suggests that recombinant 3CL has the potential to elicit neutralizing antibodies against STa toxin.

Challenge of immunized mice with ETEC

To evaluate whether 3CL antibodies can protect immunized mice against ETEC infection, immunized and non-immunized control groups were challenged with a lethal dose (1×10^5 CFU) of ETEC (CFA/I^+, LT^+, STa^+) by i.p. injection. The animals were monitored for 7 days for evidence of disease and mortality/survival rates. There was 100% mortality in the control group by 26 hr after challenge, whereas 70% of immunized mice survived (Fig. 4) after being lethargic and having diarrhea for 3 days. These data definitely support the potential of this chimeric protein to protect immunized mice from lethal doses of ETEC.

DISCUSSION

Colonization factors, which mediate bacterial attachment to intestinal epithelial cells, and LT and STa enterotoxins, which cause fluid and electrolyte hypersecretion in the small intestine, are the two main groups of ETEC virulence factors (23). Hence, there are two strategies for control of ETEC infections: blocking of bacterial adherence to CF receptors and eliminating enterotoxin activity in intestinal epithelial cells (14, 24). Inactivated whole-cell vaccines composed of *E. coli* strains that express a CF and a toxin antigen (25–28), or subunit vaccines that use a combination of CF and toxin antigens (29, 30) or a CF-toxin fusion protein (14, 31) can induce both anti-CF and anti-toxin immunity in their hosts. In other studies, integration of native STa peptide into ClpG (the major subunit of CS31A) (18) and STa toxoid into FaeG (the major

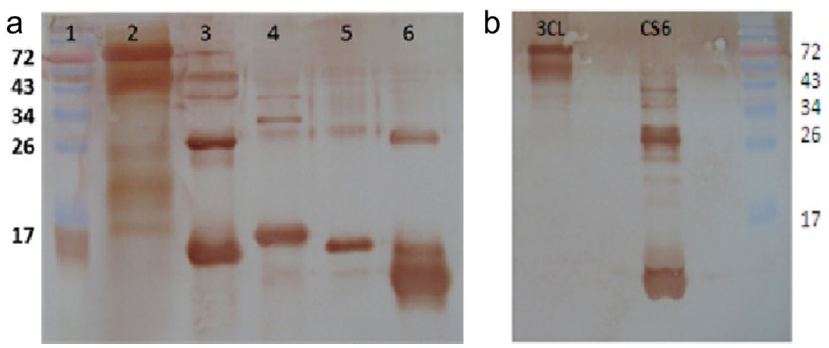

Fig. 1. Recognition of recombinant proteins by western blotting. (a) Serum specific 3CL antibody was able to recognize recombinant CfaB*ST, CssA, CssB, LTB (Lanes 3, 4, 5, 6) and (b) native CS6. Recombinant 3CL was used as positive control (Lane 2). Lane 1 is protein weight marker.

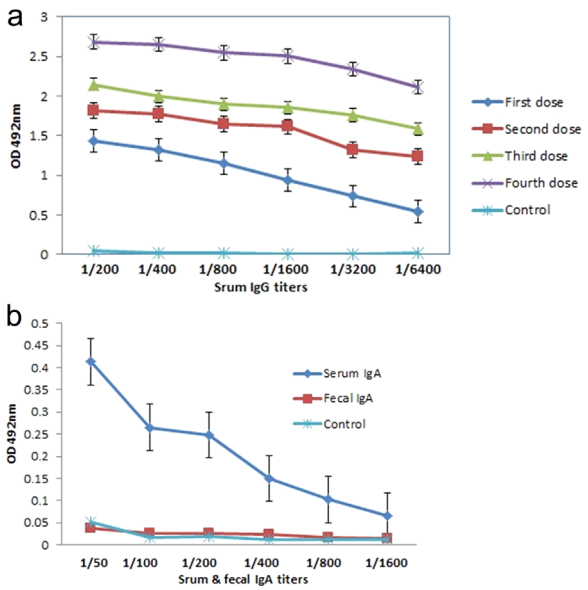

Fig. 2. 3CL-specific serum IgG and fecal IgA following s.c. immunization. Recombinant 3CL and complete and incomplete Freund's adjuvant were used for mice immunization. Immunizations were performed four times. Sera were collected after immunization and analyzed for (a) r3CL-specific IgG and (b) IgA by ELISA. Sera from non-immunized mice were used as control ($P < 0.05$).

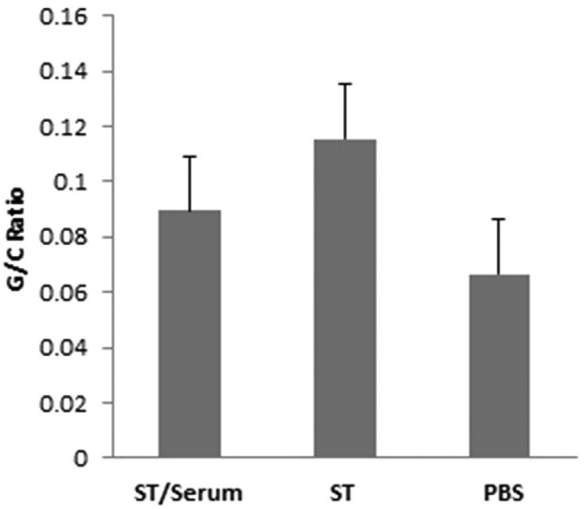

Fig. 3. Neutralizing activity of anti-3CL serum against STa in a suckling mouse assay. Sera from mice immunized with 3CL mixed with 1 MU of culture filtrate of STa-producing ETEC (40 μL) were used to inoculate newborn mice. Neutralizing activity was assessed by determining gut/carcass (G/C) weight ratios, a G/C ratio >0.090 being considered positive for STa enterotoxicity. Culture filtrates of STa-producing ETEC ($P < 0.01$) and PBS ($P < 0.03$) were used as controls. Data depict the mean ± SD of three independent experiments at each time point.

subunit of K88ac) (19), was shown to result in development of neutralizing anti-STa, anti-ClpG and anti-FaeG antibodies in immunized animal models (18, 19). These findings demonstrate that applying a couple of CFs along with LTB toxin with an embedded STa epitope can stimulate not only anti-adhesins and anti-LTB, but also anti-STa immunity. Here, we genetically integrated STa13 toxoid epitope into the CfaB major subunit of *E. coli* CFA/I fimbriae to examine a CF platform vaccine that induces both anti-STa and anti-CfaB immunity. We also fused the sequences of CssA, CssB and LTB to the carboxyl terminal of CfaB*STa sequence (20). According to studies of the toxin pattern of clinically isolated ETEC, over two-thirds of human ETEC strains are STa^+ (13). Therefore, a vaccine candidate containing an effective STa toxoid may provide approximately 60–70% protection coverage against ETEC infections (6). Expressing CfaB, CS6 structural subunits, LT and STa epitope, within CfaB structure, as a fusion protein, we produced a single antigen that can induce immunity against all of these fragments. In other words, after immunization with the chimeric recombinant protein, the mice developed antibodies that could detect each subunit in the chimeric protein (Fig. 1a,b) and also neutralize the STa toxin (Fig. 3), demonstrating the preservation of subunit proteins' conformational epitopes in the chimeric form and more importantly, displaying STa epitopes in the CfaB*STa structure.

That elicited antibodies can detect ETEC CFs and neutralize STa toxin *in vivo* does not necessarily mean that they can impede toxin loading by living bacteria. Therefore, to further strengthen our findings, we performed challenge studies to evaluate the efficiency with which these induced anti-adhesins and anti-toxin immunity provide protection against ETEC infections. Because of the reported ineffectiveness of oral challenge (32, 33), we evaluated protective antibodies against LT, STa and CFA/I producing ETEC by i.p. challenge. We found that induced anti-adhesin (anti-CfaB) and anti-toxin (both anti-LT and anti-STa) antibodies are not only neutralizing but also protective against ETEC diarrhea. The challenge route and 70% survival rate in immunized mice is comparable with the findings of Deng *et al.*, who found that mice had a 55%survival rate after i.p challenge (32), and of Yang *et al.*, who evaluated the influence of challenge routes on susceptibility to ETEC infection and concluded that intranasal (high dose) and i.p routes were effective (with 50% and 80% survival, respectively), whereas oral and intranasal (low dose) routes were ineffective in immunized mice (100% survival rate) (33).

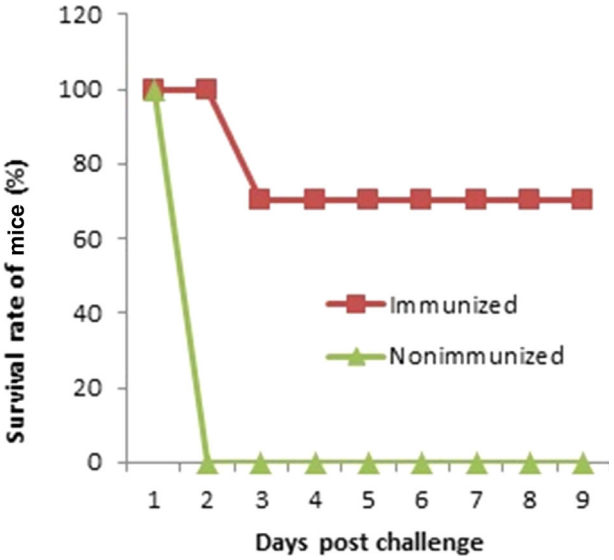

Fig. 4. Survival rate of immunized mice following ETEC challenge. Mice vaccinated with r3CL were challenged by i.p injection of a lethal dose of ETEC (CFA/I, LT+, ST+) 6 months after the initial immunization (7 days after the boosting vaccination). The number of survivors was recorded daily for 7 days following challenge.

Moreover, the endurance of the induced protection of the immunized animals (Fig. 4) 6 months after the first antigen administration clearly indicates the activation and establishment of memorial B cells, which stimulate formation of immunological memory in immunized mice (34).

According to our ELISA results (Fig. 2a,b), secretory IgA is not definitely required for the prevention of ETEC colonization in mice. In animals immunized s.c. with chimeric 3CL, CfaB, CssA/CssB and LTB-specific IgG were elicited without any evident antigen-specific IgA. There was no bacterial colonization following challenge (Fig. 4). The lack of 3CL-specific IgA in feces can reportedly be overcome by high CfaB, CssA/CssB and LTB-specific serum IgG or systemic priming of the immune system, resulting in a response following challenge (21). Recent studies have found that most IgG at mucosal surfaces is derived from the serum (35, 36). Transudation of systemic IgG antibodies appeared to be responsible for protection of immunized mice in our study.

Efficient induction of anti-STa and anti-CfaB immunity may be achieved using CfaB as a platform for STa toxoid integration in future ETEC vaccines. Comparably, Chauhan *et al.* reported that insertion of cholera toxin epitope into bacterial flagella induces of anti-cholera toxin antibodies (37). Zhang and Francis also showed that integration of detoxified STa13 epitope into the FaeG major subunit of *E. coli* K88ac fimbriae results in induction of anti-STa immunity (19). Likewise, in this investigation we selected a detoxified STa13 epitope to design CfaB*STa fusion protein. By using a suckling mouse assay, we showed that induced anti-STa immunity is capable of neutralizing native STa and inhibiting fluid accumulation in the gut of newborn mice (32) (Fig. 3).

We were unable to evaluate the anti-CS6 potential of immunized mice sera by *in vivo* assays; however, detection of native purified CS6 protein (Fig. 1b) by the immunized mice sera in immunoblot analyses strengthens the probability of sera having anti-CS6 potential in challenge studies. According to ELISA results (data not shown), anti-CssA antibody concentrations were lower than anti-CssB concentrations, which is compatible with other reports of low immunogenicity of CssA compared with CssB protein (38). Hence, we highly recommend incorporating other prevalent CF subunits rather than CssA in chimeric construct design.

We propose that *E. coli* strains expressing chimeric CFA/I fimbriae in which STa13 epitope is integrated into CfaB can be used in future live attenuated ETEC vaccine designs. It is possible that the expressed chimeric CFA/I fimbriae retains its receptor binding activity in host intestine. Given that CFA/I is one of the commonest CFs in infectious ETEC strains (39), CfaB protein also can be used as a protein carrier to increase the immunogenicity of poorly immunogenic antigens. These findings suggest that residues 74–76 of CfaB are suitable candidates for insertion of such epitopes (20).

ACKNOWLEDGMENTS

Our special thanks to Dr. Mohammad Ali Haghighi (Bushehr University of Medical Science) and the staff at the Department of Agricultural Biotechnology, NIGEB, for their kind technical assistance and Dr. Akbari for assistance in directing the suckling mouse assay. This work was supported by NIGEB grant 450.

DISCLOSURE

The authors have no financial conflicts of interest.

REFERENCES

1. Qadri F., Saha A., Ahmed T., Al Tarique A., Begum Y.A., Svennerholm A.M. (2007) Disease burden due to enterotoxigenic *Escherichia coli* in the first 2 years of life in an urban community in Bangladesh. *Infect Immun* **75**: 3961–8.
2. Hill D.R., Beeching N.J. (2010) Travelers' diarrhea. *Curr Opin Infect Dis* **23**: 481–7.
3. Fleckenstein J.M., Munson G.M., Rasko D.A. (2013) Enterotoxigenic *Escherichia coli*: Orchestrated host engagement. *Gut Microbes* **4**: 392–6.

4. Bourgeois A.L., Wierzba T.F., Walker R.I. (2016) Status of vaccine research and development for enterotoxigenic *Escherichia coli*. *Vaccine* **34**: 2880–6.
5. Madhavan T.P., Sakellaris H. (2015) Colonization factors of enterotoxigenic *Escherichia coli*. *Adv Appl Microbiol* **90**: 155–97.
6. Zhang W., Sack D.A. (2012) Progress and hurdles in the development of vaccines against enterotoxigenic *Escherichia coli* in humans. *Expert Rev Vaccines* **11**: 677–94.
7. Taxt A., Aasland R., Sommerfelt H., Nataro J., Puntervoll P. (2010) Heat-stable enterotoxin of enterotoxigenic *Escherichia coli* as a vaccine target. *Infect Immun* **78**: 1824–31.
8. Glenn G.M., Villar C.P., Flyer D.C., Bourgeois A.L., Mckenzie R., Lavker R.M., Frech S.A. (2007) Safety and immunogenicity of an enterotoxigenic *Escherichia coli* vaccine patch containing heat-labile toxin: use of skin pretreatment to disrupt the stratum corneum. *Infect Immun* **75**: 2163–70.
9. Norton E.B., Lawson L.B., Freytag L.C., Clements J.D. (2011) Characterization of a mutant *Escherichia coli* heat-labile toxin, LT(R192G/L211A), as a safe and effective oral adjuvant. *Clin Vaccine Immunol* **18**: 546–51.
10. Zhang C., Knudsen D.E., Liu M., Robertson D.C., Zhang W. (2013) Toxicity and immunogenicity of enterotoxigenic *Escherichia coli* heat-labile and heat-stable toxoid fusion 3xSTa(A14Q)-LT(S63K/R192G/L211A) in a murine model. *PLoS ONE* **8**: e 77386.
11. Zhang J., Fan H.Y., Zhang Z., Zhang J., Zhang J., Huang J.N., Ye Y., Liao M. (2016) Recombinant baculovirus vaccine containing multiple M2e and adjuvant LTB induces T cell dependent, cross-clade protection against H5N1 influenza virus in mice. *Vaccine* **34**: 622–9.
12. Ma Y. (2016) Recent advances in nontoxic *Escherichia coli* heat-labile toxin and its derivative adjuvants. *Expert Rev Vaccines* **15**: 1–11.
13. Svennerholm A.M., Lundgren A. (2012) Recent progress toward an enterotoxigenic *Escherichia coli* vaccine. *Expert Rev Vaccines* **11**: 495–507.
14. Ruan X., Sack D.A., Zhang W. (2015) Genetic fusions of a CFA/I/II/IV MEFA (multiepitope fusion antigen) and a toxoid fusion of heat-stable toxin (STa) and heat-labile toxin (LT) of enterotoxigenic Escherichia coli (ETEC) retain broad anti-CFA and antitoxin antigenicity. *PLoS ONE* **10**: e 0121623.
15. Liu M., Ruan X., Zhang C., Lawson S.R., Knudsen D.E., Nataro J.P., Robertson D.C., Zhang W. (2011) Heat-labile- and heat-stable-toxoid fusions (LTR(1)(9)(2)G-STaP(1)(3)F) of human enterotoxigenic *Escherichia coli* elicit neutralizing antitoxin antibodies. *Infect Immun* **79**: 4002–9.
16. Saarilahti H.T., Palva E.T., Holmgren J., Sanchez J. (1989) Fusion of genes encoding Escherichia coli heat-stable enterotoxin and outer membrane protein OmpC. *Infect Immun* **57**: 3663–5.
17. Rausch D., Ruan X., Nandre R., Duan Q., Hashish E., Casey T.A., Zhang W. (2016) 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). *Vet Microbiol* **202**: 78–89.
18. Batisson I., Der Vartanian M. (2000) Contribution of defined amino acid residues to the immunogenicity of recombinant *Escherichia coli* heat-stable enterotoxin fusion proteins. *FEMS Microbiol Lett* **192**: 223–9.
19. Zhang W., Francis D.H. (2010) Genetic fusions of heat-labile toxoid (LT) and heat-stable toxin b (STb) of porcine enterotoxigenic *Escherichia coli* elicit protective anti-LT and anti-STb antibodies. *Clin Vaccine Immunol* **17**: 1223–31.
20. Zeinalzadeh N., Salmanian A.H., Ahangari G., Sadeghi M., Amani J., Bathaie S.Z., Jafari M. (2014) Design and characterization of a chimeric multiepitope construct containing CfaB, heat-stable toxoid, CssA, CssB, and heat-labile toxin subunit B of enterotoxigenic *Escherichia coli*: A bioinformatic approach. *Biotechnol Appl Biochem* **61**: 517–27.
21. Amani J., Salmanian A.H., Rafati S., Mousavi S.L. (2010) Immunogenic properties of chimeric protein from espA, eae and tir genes of *Escherichia coli* O157:H7. *Vaccine* **28**: 6923–9.
22. Giannella R.A. (1976) Suckling mouse model for detection of heat-stable *Escherichia coli* enterotoxin: characteristics of the model. *Infect Immun* **14**: 95–9.
23. Fleckenstein J., Sheikh A., Qadri F. (2014) Novel antigens for enterotoxigenic *Escherichia coli* vaccines. *Expert Rev Vaccines* **13**: 631–9.
24. Nandre R.M., Ruan X., Duan Q., Sack D.A., Zhang W. (2016) Antibodies derived from an enterotoxigenic Escherichia coli (ETEC) adhesin tip MEFA (multiepitope fusion antigen) against adherence of nine ETEC adhesins: CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS21 and EtpA. *Vaccine* **34**: 3620–5.
25. Holmgren J., Bourgeois L., Carlin N., Clements J., Gustafsson B., Lundgren A., Nygren E., Tobias J., Walker R., Svennerholm A.M. (2013) Development and preclinical evaluation of safety and immunogenicity of an oral ETEC vaccine containing inactivated *E. coli* bacteria overexpressing colonization factors CFA/I, CS3, CS5 and CS6 combined with a hybrid LT/CT B subunit antigen, administered alone and together with dmLT adjuvant. *Vaccine* **31**: 2457–64.
26. Tobias J., Svennerholm A.M. (2012) Strategies to overexpress enterotoxigenic *Escherichia coli* (ETEC) colonization factors for the construction of oral whole-cell inactivated ETEC vaccine candidates. *Appl Microbiol Biotechnol* **93**: 2291–300.
27. Lundgren A., Leach S., Tobias J., Carlin N., Gustafsson B., Jertborn M., Bourgeois L., Walker R., Holmgren J., Svennerholm A.M. (2013) Clinical trial to evaluate safety and immunogenicity of an oral inactivated enterotoxigenic *Escherichia coli* prototype vaccine containing CFA/I overexpressing bacteria and recombinantly produced LTB/CTB hybrid protein. *Vaccine* **31**: 1163–70.
28. Lundgren A., Jertborn M., Svennerholm A.M. (2016) Induction of long term mucosal immunological memory in humans by an oral inactivated multivalent enterotoxigenic *Escherichia coli* vaccine. *Vaccine* **34**: 3132–40.
29. Byrd W., Cassels F.J. (2003) Mucosal immunization of BALB/c mice using enterotoxigenic *Escherichia coli* colonization factors CFA/I and CS6 administered with and without a mutant heat-labile enterotoxin. *Vaccine* **21**: 1884–93.
30. Lapa J.A., Sincock S.A., Ananthakrishnan M., Porter C.K., Cassels F.J., Brinkley C., Hall E.R., Van Hamont J., Gramling J.D., Carpenter C.M., Baqar S., Tribble D.R. (2008) Randomized clinical trial assessing the safety and immunogenicity of oral microencapsulated enterotoxigenic *Escherichia coli* surface antigen 6 with or without heat-labile enterotoxin with mutation R192G. *Clin Vaccine Immunol* **15**: 1222–8.
31. Nazarian S., Gargari S.L., Rasooli I., Hasannia S., Pirooznia N. (2014) A PLGA-encapsulated chimeric protein protects against adherence and toxicity of enterotoxigenic *Escherichia coli*. *Microbiol Res* **169**: 205–12.
32. Deng G., Zeng J., Jian M., Liu W., Zhang Z., Liu X., Wang Y. (2013) Nanoparticulated heat-stable (STa) and heat-labile B

subunit (LTB) recombinant toxin improves vaccine protection against enterotoxigenic *Escherichia coli* challenge in mouse. *J Biosci Bioeng* **115**: 147–53.
33. Yang X., Thornburg T., Holderness K., Suo Z., Cao L., Lim T., Avci R., Pascual D.W. (2011) Serum antibodies protect against intraperitoneal challenge with enterotoxigenic *Escherichia coli*. *J Biomed Biotechnol* **2011**: 632396.
34. Schmidt M.R., Mcginnes L.W., Kenward S.A., Willems K.N., Woodland R.T., Morrison T.G. (2012) Long-term and memory immune responses in mice against Newcastle disease virus-like particles containing respiratory syncytial virus glycoprotein ectodomains. *J Virol* **86**: 11654–62.
35. Meckelein B., Externest D., Schmidt M.A., Frey A. (2003) Contribution of serum immunoglobulin transudate to the antibody immune status of murine intestinal secretions: Influence of different sampling procedures. *Clin Diagn Lab Immunol* **10**: 831–4.
36. Spokesman G.M., Finn PW., Ward E.S., Dumont J., Dickinson B.L., Blumberg R.S., Lencer W.I. (2002) Receptor-mediated immunoglobulin G transport across mucosal barriers in adult life: functional expression of FcRn in the mammalian lung. *J Exp Med* **196**: 303–10.
37. Chauhan N., Kumar R., Badhai J., Preet A., Yadava P.K. (2005) Immunogenicity of cholera toxin B epitope inserted in Salmonella flagellin expressed on bacteria and administered as DNA vaccine. *Mol Cell Biochem* **276**: 1–6.
38. Ghosal A., Bhowmick R., Banerjee R., Ganguly S., Yamasaki S., Ramamurthy T., Hamabata T., Chatterjee N.S. (2009) Characterization and studies of the cellular interaction of native colonization factor CS6 purified from a clinical isolate of enterotoxigenic *Escherichia coli*. *Infect Immun* **77**: 2125–35.
39. Gaastra W., Svennerholm A.M. (1996) Colonization factors of human enterotoxigenic *Escherichia coli* (ETEC). *Trends Microbiol* **4**: 444–52.