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Murine immunization with CS21 pili or LngA major subunit of enterotoxigenic *Escherichia coli* (ETEC) elicits systemic and mucosal immune responses and inhibits ETEC gut colonization

Chengxian Zhang, Junaid Iqbal, and Oscar G. Gómez-Duarte*

Division of Infectious Diseases, Department of Pediatrics, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

Abstract

CS21 pili of enterotoxigenic *Escherichia coli* (ETEC) is one of the most prevalent ETEC colonization factors. CS21 major subunit, LngA, mediates ETEC adherence to intestinal cells, and contributes to ETEC pathogenesis in a neonatal mouse infection model. The objectives of this work were to evaluate LngA major subunit purified protein and CS21 purified pili on immunogenicity and protection against ETEC colonization of mice intestine. Recombinant LngA purified protein or purified CS21 pili from E9034A ETEC strain were evaluated for immunogenicity after immunization of C57BL/6 mice. Specific anti-LngA antibodies were detected from mice serum, feces, and intestine fluid samples by ELISA assays. Protection against gut colonization was evaluated on immunized mice orally challenged with wild type E9034A ETEC strain and by subsequent quantification of bacterial colony forming units (CFU) recovered from feces. Recombinant LngA protein and CS21 pili induced specific humoral and mucosal anti-LngA antibodies in the mouse model. CS21 combined with CT delivered intranasally as well as LngA combined with incomplete Freund adjuvant delivered intraperitoneally inhibited ETEC gut colonization in a mouse model. In conclusion, both LngA purified protein and CS21 pili from ETEC are highly immunogenic and may inhibit ETEC intestinal shedding. Our data on immunogenicity and immunoprotection indicates that CS21 is a suitable vaccine candidate for a future multivalent vaccine against ETEC diarrhea.

Keywords

Enterotoxigenic *Escherichia coli* (ETEC); CS21; type IV pili; LngA; vaccine; mucosal immunity; colonization

*Corresponding author: Division of Infectious Diseases, Department of Pediatrics, Vanderbilt University School of Medicine, 1161 21st Avenue South, Suite D6201 Medical Center North, Nashville TN, 37232-2581, USA; Tel.: +1 6153222250; Fax: +1 6153437659; oscar.gomez@Vanderbilt.Edu.

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Conflict of Interest

None

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) are a leading cause of traveler's diarrhea (Leung et al. 2006) and childhood morbidity and mortality in low-income countries and there is no vaccine available for ETEC diarrhea prevention at this time (Kotloff et al. 2013). ETEC gut colonization by coli surface antigens (CSs) is crucial for pathogenicity and CS21 (a.k.a. longus for long pilus), is one the most prevalent CSs among worldwide ETECs. CS21, toxin-co-regulated pilus (TCP) of *Vibrio cholerae*, bundle-forming pilus (BFP) of enteropathogenic *E. coli* and CS8 (CFA/III) of ETEC are class-B type-4 pili (Girón et al. 1997). A 16-gene cluster, essential in the biosynthesis of CS21, includes structural and regulatory genes (Gomez-Duarte et al. 2007). The CS21 structural subunit gene *lngA* encodes a 22 kDa mature protein implicated in CS21-mediated ETEC specific adherence to intestinal cells (Guevara et al. 2013). CS21 also mediated bacterial self-aggregation believed to promote resistance to environmental stress factors (including antimicrobials) (Clavijo et al. 2010, Cruz-Córdova et al. 2014, Guevara et al. 2013, Mazariego-Espinosa et al. 2010).

Latin America and the Caribbean as well as the Middle East and North African geographic regions have the highest prevalence of CS21 positive ETEC clinical isolates. Among Latin American countries, Chile has the highest proportion of CS21 positive ETEC strains with a prevalence ranging from 38 to 71.8% (Del Canto et al. 2012a, Girón et al. 1995). The prevalence of CS21 positive ETECs in Colombia, Brazil, Bolivia and Argentina was 50%, 32.8%, 23.2%, and 17%, respectively (Guerra et al. 2014, Nada et al. 2011, Pichel et al. 2002a). The prevalence of CS21 positive ETECs in African and Asian countries including Egypt, South Korea and Bangladesh was 15%, 19.9%, and 19%, respectively (Nada et al. 2011, Oh et al. 2014a, Pichel et al. 2002a). Spanish travelers with diarrhea reported CS21 in 58% of ETEC isolates (Rivera et al. 2013). The high prevalence of CS21 among ETEC clinical isolates globally makes this colonization factor an important antigen to be included in a polyvalent vaccine against ETEC diarrhea (Isidean et al. 2011).

ETEC CSs are highly immunogenic and are promising components of a multivalent ETEC vaccine (Sjöling et al. 2015, Svennerholm & Tobias 2008, Zhang & Sack 2012). Unfortunately, knowledge on CS21 immunogenicity and immunoprotection is limited and none of the previously described ETEC vaccines have included CS21 antigens. Consequently, it is imperative to investigate the vaccine potential of CS21 with respect to immunogenicity and protection against ETEC intestinal colonization. We hypothesize that CS21 and LngA are highly immunogenic and able to induce specific anti-CS21 antibodies. Anti-LngA antibody binding to CS21 inhibits CS21-mediated ETEC adherence to intestinal epithelial cells and protects against ETEC intestinal colonization.

This study was conducted to demonstrate that CS21 and LngA antigen delivery to a mammalian host by different routes may induce specific and protective immune responses. We showed that both, LngA and CS21, are highly immunogenic and that CS21 immunogen can inhibit ETEC colonization. Our studies on immunogenicity and immunoprotection place CS21 in the list of suitable vaccine candidates for a future multivalent vaccine against ETEC diarrhea.

Materials and Methods

Bacterial strains

Bacterial strains used in this study are described in Table 1. *E. coli* DH5 α (Life Technologies, Grand Island, NY), and CS21⁺ or CS8⁺ ETEC strains were cultured on Luria broth (LB), Terrific broth (TB) or TB agar plates at 37 °C overnight (Clavijo et al. 2010). CS21⁺ ETEC E0934A strain was electroporated with plasmid pCM17 containing luciferase genes (Morin & Kaper 2009, Rhee et al. 2011). The pCM17 was kindly provided by Dr. James B. Kaper. E0934A/pCM17, used for *in vivo* challenge mice experiments, was cultured in LB broth supplemented with Kanamycin at 37 °C overnight and then sub-cultured in DMEM/F12 (1:1) medium (25 mM glucose, 15 mM HEPES and 0.5% mannose) at ratio of 1:10 for 3 hours at 37 °C. The bacteria were washed once with sterile PBS and the bacterial concentration was adjusted in sterile PBS before challenge.

LngA and CS21 isolation and purification

The recombinant LngA-His protein was prepared by GenScript (Piscataway, NJ). Briefly, the LngA gene derived from wild-type ETEC E9034A along with the 6xHis tag DNA sequence were synthesized and subcloned into pET15b vector. *E. coli* BL21 (DE3) was transformed by the recombinant plasmid and induced by IPTG to overexpress LngA-His recombinant protein. Bacteria culture was harvested by centrifugation, lysed by sonication, and the lysate supernatant was subject to purification by a Ni-NTA column. CS21 or CS8 pili were purified as described before from wild-type ETECs (Girón et al. 1994). Briefly, wild-type ETECs were cultured on TB agar plates overnight at 37°C, harvested in sterile phosphate buffer solution (PBS), vortexed for one minute, and centrifuged at 8,000 $\times$ g and 4 °C for 20 minutes. The supernatant was centrifuged at 12,000 $\times$ g and 4 °C for 30 minutes and subsequently centrifuged at 40,000 $\times$ g at 4 °C for 30 minutes. The pellet containing CS21 or CS8 pili was resuspended in sterile PBS and stored at -80°C until use. The protein concentration was determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions.

Multiple protein sequence alignment and B cell epitope prediction

A multiple protein sequence alignment was generated by the DNADynamo software (<http://www.bluetractorsoftware.co.uk/>) by using LngA amino acid sequences derived from CS21⁺ ETEC strains E9034A (NG_036490.1), M526-C6B (EU107107.1), BR5 (EU107105.1), MP215-1 (EU107104.1), M633-C1 (EU107103.1), M626-C (EU107102.1), M452-C1 (EU107101.1), M445-C1 (EU107100.1), G1026 (EU107099.1), B7A (EU107098.1), 2108-2 (EU107097.1), 11381a (EU107096.1), 10159a (EU107095.1), M408 (EU107094.1), P307 (EU107093.1), M424-C1 (EU107092.1), M145-C2 (EU107091.1), B2C (EU107090.1), 01117-5 (EU107089.1). The GeneBank accession numbers for CofA, BfpA, and TcpA proteins are CEJ09700.1, WP_000253758.1, and AAL58332.1, respectively. The linear B cell epitopes of LngA major subunit derived from strain E9034A were predicted by Bepipred Linear Epitope Prediction (Larsen et al. 2006), Parker Hydrophilicity Prediction (Parker et al. 1986), and Emini Surface Accessibility Prediction (Emini et al. 1985) of Antibody Epitope Prediction on IEDB Analysis Resources (<http://tools.immuneepitope.org/bcell/>).

Immunogenicity experiments

All animal experiments performed in this study were approved by the IACUC at Vanderbilt University under protocol M/11/223. For LngA immunogenicity studies, twenty-five 7- to 8-weeks old C57BL/6 mice were purchased from Jackson Laboratories and divided into five groups with five mice per group. Mice in each group received by intraperitoneal injection a combination of one antigen or placebo plus one adjuvant (1:1, v/v): group A: PBS plus incomplete Freund's adjuvant (IFA) (Sigma, St. Louis, MO); group B: 30 µg LngA plus IFA; group C: PBS plus aluminum hydroxide (ALUM), (InvivoGen, San Diego, CA); group D: 30 µg LngA and Alum; group E: 30 µg LngA and 2 µg cholera toxin (CT) per mouse. Mice were immunized three times by intraperitoneal injection at two-week intervals. Blood and fecal samples were collected one day before each immunization and 10 days after the final immunization by submandibular vein or natural defecation, respectively. Serum samples were obtained after blood samples were clotted and centrifuged. Fecal samples were weighed, suspended in reconstitution buffer (10 mM Tris, 100 mM NaCl, 0.05% Tween-20, 5mM sodium azide, pH 7.4) supplemented with 1x protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA) at 5:1 ratio (5 ml reconstitution buffer per gram of feces) and centrifuged. The clear fecal antibody suspension in the supernatant was collected and stored at -20°C for further studies. In addition to serum and stools, intestinal fluid samples were also collected after the mice were euthanized. One gram of intestinal fluid was homogenized with 2.5 mLs of reconstitution buffer with 1x protease inhibitor cocktail and centrifuged. The cleared intestinal fluid in the supernatant was collected and stored at -20°C until used.

Immunoprotection experiments

Mice were immunized with either LngA-His recombinant protein or purified CS21 pili as described above and then challenged with the wild-type CS21-expressing ETEC E9034A strain to evaluate for protection against intestinal colonization by ETEC. Two independent experiments were conducted, one tested LngA purified protein and another tested native CS21 purified pili. In the LngA immunoprotection study, 7- to 8-week old C57BL/6 mice were divided into four groups with five mice per group. Each group received different combinations of vaccine or placebo plus adjuvants by intraperitoneal injection: group A: no vaccine (naïve group); group B: PBS and IFA (1:1, v/v); group C: 30 µg LngA and IFA (1:1, v/v); group D: 30 µg LngA and 2 µg CT per mouse. For CS21 immunoprotection study, forty-five 7- to 8-week old C57BL/6 mice were divided into nine groups with five mice per group. Each group received different combinations of vaccine or placebo plus adjuvants by subcutaneous (SC), intranasal (IN), or intraperitoneal (IP) route: group A: PBS and ALUM (1:1, v/v) in 200 µl volume by the SC route; group B: 50 µg CS21 and ALUM (1:1, v/v) in 200 µl volume by the SC route; group C: PBS and 2 µg CT in 20 µl volume by the IN route; group D: 20 µg CS21 and CT in 20 µl volume by the IN route; group E: PBS and IFA (1:1, v/v) in 300 µl volume by the IP route; group F: 50 µg CS21 and IFA (1:1, v/v) in 300 µl volume by the IP route; group G: PBS and ALUM (1:1, v/v) in 300 µl volume by the IP route; group H: 50 µg CS21 and ALUM (1:1, v/v) in 300 µl volume by the IP route; group I: no vaccine (naïve group). For antibody titration, blood and fecal samples were collected before the primary immunization and seven days after the final immunization and the intestine wash samples were collected after euthanasia as described above.

Eight days after final immunization, each group of mice were given streptomycin (5g/L) for 24 hours and were switched to regular water for 24 hours. The purpose of the streptomycin was to depopulate mice intestine normal flora and facilitate the ETEC challenge strain colonization of the mouse intestine (Rhee et al. 2011, Roy et al. 2009). Ten days after the final immunization, all groups except the naïve group (no vaccine group) were challenge by oral gavage with 1×10^8 or 1×10^6 CFU wild-type ETEC strain E9034A carrying luciferase gene plasmid pCM17 for LngA or CS21 immunoprotection study, respectively. *In vivo* bioluminescence imaging was carried out by *in vivo* imaging system XENGEN IVIS 200 (Xenogen Corporation, Alameda, California) before challenge and daily after challenge to quantify the number of colonizing bioluminescent ETEC.

In addition to measuring bioluminescence to evaluate the ETEC colonization level, ETEC colony forming units (CFU) were also calculated from fecal samples collected daily from each mice after challenge. Bacterial shedding is used as a murine marker of intestinal colonization. Accordingly, feces were collected, weighed, suspended and diluted in sterile PBS, and plated on MacConkey agar to determine the *E. coli* CFU/g feces.

ELISA assays

Indirect ELISA assays were used for titration of anti-LngA and anti-CT antibodies from serum, fecal, and intestinal samples of immunized mice. CS21 pili were coated on ELISA plates to titrate anti-LngA antibody-containing sera from mice immunized with LngA-His recombinant protein. Similarly, LngA-His protein was coated on ELISA plates to titrate anti-CS21 antibody-containing sera from mice immunized with CS21. These coating strategies would minimized the recognition of antibodies not directed to CS21 or to LngA, including anti-His antibodies. Briefly, Immulon 2 HB 96 well microtiter plates (Thermo Fisher Scientific, Waltham, MA) were coated in duplicates with 25 ng of purified CS21 or LngA, 500 ng of purified CS21 or LngA, or 500 ng of purified CT (Sigma-Aldrich, St. Louis, MO) in 100 μ l Antigen Coating Buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6) and incubated at 37 °C for one hour. Plates were washed three times with 200 μ l PBS supplemented with 0.05% Tween-20 (PBS-T) per well and blocked with 10% skim milk/PBS-T at 37 °C for one hour and washed three times with 200 μ l PBS-T per well. Serum, fecal, and intestinal wash antibody samples were serially diluted in PBS-T (fecal samples) or 5% skim milk/PBS-T (serum and intestine wash samples) and incubated at 37 °C for one hour. Plates were washed three times with 200 μ l PBS-T per well. The horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (1:5000) or IgA (1:1000) diluted in PBS-T was added to each well and plates were incubated at 37 °C for one hour. Plates were washed three times with 200 μ l PBS-T per well. One hundred microliters 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (KPL, Gaithersburg, Maryland) were added to each well to incubate at room temperature for 20 minutes and 100 μ l stop solution were added to stop the reaction. The optical density (OD) value was measured at 450 nm. The antibody titers were presented as the log₁₀ value of the antibody dilution when OD_{450nm} was 0.4. Samples with OD_{450nm} less than 0.4 are considered as negative.

Polyacrylamide gel electrophoresis and Immunoblotting

LngA-His recombinant protein was prepared by GenScript (Piscataway, NJ) and CS21 and CS8 pili were purified from ETEC strains of different countries as described above. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels were stained with Coomassie blue and processed for immunoblotting with anti-LngA monoclonal antibody or mice antisera from this immunization study. Pili preparations of laboratory reference strain DH5a and CS21⁺ ETEC strain E9034A were used as negative and positive controls and LngA-His recombinant protein was used as purified protein control. Pili and LngA-His recombinant proteins were separated by electrophoresis on pre-casted 4–20% SDS-PAGE gel (Bio-Rad, Hercules, CA) and were either stained with Bio-Safe Coomassie G-250 stain (Bio-Rad, Hercules, CA) for one hour and then washed with water or transferred to nitrocellulose membrane by Semi-dry Blotting Unit Electrophoresis System (Thermo Fisher Scientific, Waltham, MA). The nitrocellulose membrane was blocked with 10% skim milk/PBS-T for one hour, washed three times with PBS-T, probed with anti-LngA monoclonal antibody or mice antisera (derived from CS21 and LngA immunization study) in 2% skim milk/PBS-T for one hour, washed three times with PBS-T, probed by IRDye® 680LT goat anti-mouse IgG (LI-COR, Lincoln, NE) for one hour, washed three times with PBS-T, and scanned by the LI-COR Odyssey Infrared Imaging System at 700 nm channel.

Statistical analysis

Statistical analysis of data were conducted by GraphPad Prism 6 (San Diego, CA) using Fisher test. *P*-values less than 0.05 were considered statistically significant.

Results

Variable regions within the LngA protein overlap with surface accessible B cell epitopes

Two major variable regions were identified within the LngA protein sequence when 19 CS21⁺ ETEC strains LngA sequences were aligned and compared (Figure 1A). The variable regions spanned from residues 56 to 77 and 148 to 197. Cysteines at LngA residues 132 and 194 were conserved among all LngA variants (Figure 1A). Equivalent disulfide-bond-forming cysteines were identified among homologous pili major subunits including CofA (ETEC CS8) residues 131 and 193, BfpA (EPEC) residues 129 and 179, and TcpA (*Vibrio cholerae*) residues 120 and 186 (Figure 1B). Linear B cell epitopes (Figure 2A), hydrophilicity regions (Figure 2B), and surface accessibility sequences (Figure 2C) were identified within the LngA sequence. Five potential surface accessible and hydrophilic B cell epitopes were identified at residues ⁵⁰AYQRDGKYPDFV⁶¹, ⁷⁰TIKTDTS⁷⁹GIP⁷⁹, ⁸⁹ITPDEV⁹⁷RNN⁹⁷, ¹⁰⁹LTSNGA¹¹⁷QV¹¹⁷, ¹⁷⁸GNNGQT¹⁸⁶TTLT¹⁸⁶, and tentatively designated as epitopes 1 to 5 (EP1 to 5), respectively (Figure 1A and Figure 2). Predicted epitopes 1, 2 and 5 contained 2, 4 and 3 single amino acid polymorphisms, respectively (Figure 1A).

LngA-His recombinant and CS21 protein preparations stimulated murine anti-LngA antibodies cross-reacts with CS21 and CS8 homologues

LngA-His recombinant protein and CS21 native pili derived from E904A ETEC strain were analyzed by SDS-PAGE to confirm protein purity and stability before immunization experiments. LngA-His recombinant and CS21-derived native LngA proteins were visualized in the Coomassie stained gel and they were specifically recognized in Western blot by anti-LngA monoclonal antibody (Figure 3A). Coomassie stained gels of denatured CS21 purified pili showed not only the LngA pilin (22 kDa) subunit but also an unknown 70kDa protein. Based on band size and intensity, both proteins were present at similar concentrations (Figure 3A). Although the 70 kDa unknown protein was not recognized by the anti-LngA monoclonal antibody, it is unique to ETEC, since it was also produced by the *E9034A lngA* mutant strain and it was not produced by the *E. coli* BL21 strain (Figure 3A).

To test for anti-LngA antibody cross-reactivity with CS21 or CS8 major subunits, worldwide ETEC strains expressing CS21 or CS8 major subunit alleles were tested by immunoblot using mice serum raised against LngA and CS21 protein derived from CS21⁺ ETEC E9034A strain (Table 1). Pili preparation of laboratory reference *E. coli* DH5 α and CS21⁺ ETEC E9034A strains were used as negative and positive controls for LngA immunoblotting, respectively. LngA-His recombinant protein was used as protein positive control. The proteins were probed by anti-LngA mice sera. As shown in Figure 3B, anti-LngA antibody raised against LngA-His and CS21 pili derived from E9034A had strong reaction with LngA derived from CS21⁺ ETEC strains E9034A (Caribbean), 10159-a (Chile), COCt129 (Colombia), COCt40 (Colombia), M452-C1 (Morocco), and B2C (Vietnam) and weak reaction to LngA of B7A (Vietnam). Weak signal was also observed for the CofA major subunit of CS8⁺ ETEC strains E2528C1 (Caribbean) and WS6866B-2 (Egypt). The only protein no recognized by anti-LngA sera was CofA from ETEC M403-C3 ETEC (Bangladesh) strain.

Both LngA and purified CS21 pili elicit strong anti-LngA antibody responses

To evaluate CS21 pili and LngA major subunit immunogenicity, we immunized C57BL/6 mice with purified CS21 pili or LngA-His recombinant protein by subcutaneous (SC), intranasal (IN), or intraperitoneal (IP) injection and titrated specific anti-LngA antibodies in serum, fecal, and intestinal wash samples by ELISA. Mice immunized by IP route with LngA plus incomplete IFA, ALUM, and CT mounted significant anti-LngA serum IgG responses (titers (Log10)=5.6, $p<0.01$; 5.8, $p<0.01$; 6, $p<0.01$, respectively) compared with mock immunized controls (Figure 4A). The serum IgG responses from mice immunized with LngA plus three different adjuvants were seen as early as 13 days after the primary immunization and increased steadily overtime until reaching a peak response at 37 days after primary immunization (Figure 4D). In addition, mice immunized with LngA plus IFA, ALUM, and CT mounted significant anti-LngA fecal IgA (titers (Log10) =1.0, $p=0.05$; 0.8, $p=0.04$; 1.8, $p<0.01$, respectively) (Figure 4B) and intestinal wash IgA responses (titers (Log10) =2.3, $p<0.01$; 1.8, $p<0.01$; 2.6, $p<0.01$, respectively) (Figure 4C).

Mice immunized with CS21 plus ALUM by SC route, CS21 plus CT by IN route, CS21 plus IFA by IP route, and CS21 plus ALUM by IP route mounted significant anti-LngA serum

IgG responses (titers (Log10) = 4.8, $p<0.01$; 2.4, $p<0.01$; 5.7, $p<0.01$; 4.1, $p<0.01$ respectively) compared with mock immunized controls (Figure 5A). In addition, mice immunized with CS21 mounted significant anti-LngA fecal IgA (titer (Log10) of CS21 plus IFA by IP=2.9, $p=0.0116$; titer (Log10) of CS21 plus ALUM by IP=2.4, $p=0.0123$, respectively) (Figure 5B) and intestinal wash IgA responses (titers (Log10) =1.4, $p<0.01$; 0.4, $p=0.3466$; 3.8, $p<0.01$; 2.4, $p<0.01$ respectively) (Figure 5C).

Since CT is not only a potent adjuvant but an immunogen, when delivered as adjuvant with CS21 or LngA, it stimulated strong serum anti-CT IgG, fecal IgA, and intestinal wash IgA antibody responses (Figure 5D).

LngA and CS21 immunization inhibits wild-type ETEC gut colonization in mice

To evaluate the role of CS21 and LngA in immunoprotection against mice gut colonization, mice immunized with LngA protein or with CS21 pili were orally challenged with CS21⁺ wild type ETEC E9034A strain and subsequently evaluated for gut colonization by quantifying bacteria shedding from stools.

For mice immunized IP with LngA, the LngA+IFA immunized group shed less bacteria in feces 5 days after challenge than the negative control group or the LngA+CT group (Figure 6A). LngA+IFA immunized group stopped shedding bacteria in the stools from day 11 after challenge. For mice immunized IN with CS21, the CS21+CT immunized group shed significantly less bacteria in feces than the unimmunized control or the remaining immunized groups (Figure 6B). The CS21+CT IN immunized group stopped shedding bacteria in the stools from day 12 after challenge. In contrast, all remaining CS21 immunized groups and controls continue shedding bacteria in the stool after day 16 (Figure 6 panels C–E). Differences in bacterial shedding in mice immunized SC or IP with CS21+IFA or CS21+ALUM. were only significant at certain days after challenge (Figure 6 panels C–E). In summary, IP LngA+IFA and IN CS21+CT vaccine strategies resulted in lower fecal bacterial shedding after ETEC oral challenge of C57BL/6 mice.

Discussion

ETEC isolates reaching the gastrointestinal tract via oral transmission, bind to intestinal epithelial cells through CSs, and colonize the intestinal mucosal surface (Porter et al. 2011). CSs-based vaccines may elicit specific immune responses that by blocking CSs-mediated ETEC adherence to intestinal cells they may inhibit ETEC gut colonization, and prevent diarrhea. Unlike other CSs such as CFA/I, CS1, CS5, and CS8 which have been intensively studied as potential vaccine candidates, the immunogenicity and immunoprotective activity of CS21 and its major subunit LngA have not been explored. This study showed that native CS21 and LngA recombinant proteins are highly immunogenic in the murine model and they elicit a robust systemic IgG and mucosal secretory IgA responses. Under unique delivery and adjuvant conditions, LngA and CS21 inhibited ETEC gut colonization measured by the lower fecal shedding among immunized mice compared to controls. This suggested that anti-CS21 and anti-LngA immune responses may inhibit CS21-mediated ETEC binding to intestinal cells and may block gut colonization. Evidence that anti-LngA

antibodies inhibited CS21-dependent ETEC adherence to intestinal cells was previously demonstrated *in vitro* (Guevara et al. 2013).

ETEC CSs have been tested as vaccines to protect against ETEC colonization and diarrheal diseases in various forms including live attenuated bacteria, inactivated bacteria, and protein subunits. In one vaccine study in animals, attenuated ETEC strains expressing the CFA/I and a detoxified heat-labile enterotoxin (LT_hK63) enhanced clearance of ETEC from the lungs of mice and protected mice from intestinal ETEC colonization and LT-induced fluid accumulation (Byrd & Boedeker 2013). In one human volunteer study, a three-strain-combination live attenuated vaccine known as ACE527 was created to include strain ACAM2025 expressing CFA/I and LT_B; strain ACAM2022 expressing CS5, CS6, and LT_B; and strain ACAM2027 expressing CS1, CS2, CS3, and LT_B. This vaccine was well tolerated and at least 50% of subjects in the high-dose group responded to LT_B, CFA/I, CS3, and CS6 (Harro et al. 2011). In addition, it reduced the incidence and severity of diarrhea in a human challenge model of diarrheal disease (Darsley et al. 2012). In preclinical vaccine development studies, oral inactivated ETEC prototype vaccines producing CFA/I or CFA/I +CS3+CS5+CS6 and recombinant LT_B/CT_B hybrid protein were shown to be safe and well tolerated and stimulated significant systemic and mucosal immune responses to each of the vaccine CF antigens as well as to LT B subunit (Holmgren et al. 2013, Lundgren et al. 2013). In one ETEC subunit vaccine study, epitopes from seven CS were genetically fused to STa-LT toxoid constructs to create multi-epitope fusion antigens (MEFAs) CFA/I/II/IV-STaA14Q-dmLT and CFA/I/II/IV-STaN12S-dmLT. Antibodies induced by the MEFAs showed *in vitro* adherence inhibition activities against ETEC or *E. coli* strains expressing these seven CFAs and neutralization activities against both toxins (Ruan et al. 2014). Based on our immunoprotection data and previously published studies reporting high global prevalence of CS21 among ETEC strains, we believe that CS21 should be included as an antigen in new polyvalent ETEC vaccines to tested for protection against toxin-mediated diarrhea in the human host.

Antigenic variations were observed at the predicted LngA surface accessible B-cell epitopes. While LngA antigenic variation may be a critical mechanism to evade host memory immune responses and facilitate the re-infection (Kotwal & Kulkarni 2001), the number of B-cell epitopes was high enough to allow for antigen recognition among different LngA variants. Anti-LngA antibodies have strong cross-reactivity with genetically diverse LngA proteins and weak cross-reactivity with evolutionally related CS8 pili, as demonstrated by immunoblotting analysis. This indicates that anti-LngA antibody has the potential to provide broad protection against ETECs expressing type 4 pili cross-reactive variants.

Anti-LngA antibodies are not the only explanation for the protection against gut colonization observed with LngA+IFA vaccine given IP or the CS21+CT vaccine given IN. Interestingly, all antigen-adjuvant combinations delivered by any route were successful in eliciting strong and specific anti-LngA or anti-CS21 antibody responses yet only two vaccine combinations -LngA+IFA given IP or the CS21+CT given IN- were associated with protection. Since no protection against colonization was observed with LngA+CT given IP, or with CS21+ALUM or CS21+IFA given IP or SC, we believe that specific anti-LngA or anti-CS21 non-antibody immune responses should contribute to protection against colonization.

Further experiments are necessary to elucidate the specific factors involved in protection when using LngA or CS21 as immunogens. Comparing LngA and CS21 head to head or in combination with different adjuvants and by different delivery routes may provide critical information on how to optimize LngA or CS21 immunoprotection.

Overall, IN delivery of CS21 pili in combination with CT outperformed recombinant IP delivery of LngA plus IFA in protecting mice against ETEC intestinal colonization based on reduced fecal ETEC shedding. One explanation for better protection with CS21 compared with LngA is that recombinant LngA protein may have altered tertiary structures with respect to the native CS21 LngA conformation. As a consequence, anti-LngA antibodies derived from recombinant LngA may react less efficiently against the native LngA protein in CS21 compared with those derived from purified CS21 pili. An additional explanation for better protection with CS21 pili may have to do with CS21+ ETEC co-purified proteins. A 70kDa protein (Figure 5A) and other subunits of CS21 pili, e.g. minor pilin subunit LngB, may contribute additional protection compared to LngA alone against ETEC challenge and colonization. The role of the unknown 70kDa protein and LngB in stimulation of protective immune responses is yet to be defined. We can't rule out that additional ETEC surface protein traces co-precipitated in the CS21 preparation may also contribute to immunoprotection. CS3, among them is expressed by E9034A ETEC strain (Levine et al. 1984) and it is also associated with cell adherence to intestinal epithelial cells.

LngA- and CS21 immunized mice were challenged with bioluminescent ETEC and the intestinal bacterial load was evaluated by measuring bioluminescence. During the first three days of daily measurements there were no significant differences between control and immunized groups (data not shown) and days later the bioluminescence signal was very low or undetectable. Limited bioluminescence detection may explain the low sensitivity of detection in this colonization model. Also, the high challenge dose of 1×10^8 CFU per mice, may have overwhelmed mucosal immune responses and lead to high ETEC colonization in both control and immunized groups with no appreciable differences. In contrast, CFU detected in feces was a more sensitive method of measuring ETEC intestinal colonization as CFU shedding remained positive one week after bioluminescence signal became negative. By measuring CFU it was shown that ETEC were cleared in LngA-immunized mice 11 days post challenge, suggesting that immunized mice are more prone to clear ETEC colonization from mice intestines after intestinal ETEC load decreases over time. One limitation of this quantitative assay is that ETEC stool shedding may not accurately indicate the level of ETEC colonization in the small or large intestines. Future *in vivo* experiments may need to address the level of ETEC gut colonization after oral infections and the protection against gut colonization by alternative techniques, including more sensitive *in vivo* bioluminescence detection imaging or by directly quantifying intestinal content in immunized and unimmunized animals.

This study provides evidence on how ETEC CSs are immunoprotective (Qadri et al. 2005, Svennerholm & Tobias 2008, Tobias & Svennerholm 2012, Zhang & Sack 2012) and emphasizes the need for a multivalent vaccine for ETEC diarrhea prevention. An ideal ETEC vaccine should include the most prevalent CSs to provide the maximum protection against highly heterogeneous ETEC populations. At least 23 specific coli surface antigens

(CSs) have been identified in human ETEC strains and it is not unusual for one ETEC strain to express two or more types of CSs at the same time (Del Canto et al. 2012b, Gaastra & Svennerholm 1996, Pichel et al. 2000). Although the better-performing CS21 pili conferred appreciable protection against ETEC colonization on several days post challenge, the immunized mice were still colonized with ETEC at lower levels. We predict that anti-CS21 antibodies prevent ETEC binding to intestinal cell surface and ETEC floating on the mucus surface are unable to effectively deliver LT/ST toxins to cells to induce disease. The limited number of mice per group in our animal experiments may have limited the power of the study during statistical analysis. More preclinical studies with higher number of mice per group are necessary to evaluate immunoprotection against ETEC colonization. Furthermore, we propose to use better animal models, including the *Aotus nancymae* animal model to not only test colonization but also diarrheal disease. This animal model was reported to reproduce colonization and diarrheal disease in human by ETEC and *Campylobacter spp.* (Jones et al. 2006a,b).

Immunization routes and adjuvants appear to play roles in eliciting immune responses and inhibiting ETEC colonization and fecal shedding. Immunizations via intraperitoneal route elicited the highest level of anti-LngA antibody responses and showed more inhibitory effects against ETEC colonization and fecal shedding. Unfortunately, intraperitoneal immunization is not practical in human vaccination. Mice immunized IN with CS21 mounted moderate antibody responses, yet, they cleared ETEC more efficiently after 7 days post challenge, indicating mucosal delivery of CSs may be important for vaccine design. IFA, a potent pro-inflammatory agent, outperformed ALUM in stimulating antibody responses and inhibiting ETEC colonization, yet ALUM, because of safety, is a preferred adjuvant in human vaccines (Aucouturier et al. 2001, Gupta et al. 1993). Heat-labile enterotoxin (LT), a closely related toxin to cholera toxin, has been shown to provides a significant advantage in colonization of the small intestine *in vitro* and the mouse model (Allen et al. 2006, Johnson et al. 2009). Therefore, it is reasoned that anti-LT or anti-CT immunity may promote anti-colonization immunity. Co-administration of CS21 and adjuvant CT provided significant inhibitory effects on bacteria feces shedding (Figure 6C). This data suggest a synergistic effect on immunoprotection when CT adjuvant and CS antigens are used as immunogens. It is also likely that immune responses other than antibodies may also play roles in ETEC infection (Ren et al. 2014). Different antigens and adjuvant combinations, and immunization routes may elicit different immune responses profiles and, as a result, different protection outcomes. New studies should be conducted to optimize vaccine formulation and dosage, immunization route, adjuvant, and schedule to stimulate strong and protective anti-CS21 immunity. Continued research on CS21 vaccines may pave the way to vaccine research directed to the development innovative polyvalent safe and immunogenic ETEC vaccines that may reach phase I clinical trials.

In summary, LngA and CS21 elicit strong and specific systemic and mucosal antibody responses in mice. More importantly, we have demonstrated that LngA and CS21 immunization, in combination with specific adjuvants and delivered routes, inhibit ETEC colonization of the mice gut. CS21 is a highly prevalent CS among ETEC worldwide and immune responses against CS21 may contribute to protection against ETEC diarrhea in the mammalian host. Further immunoprotection preclinical studies are necessary to define the

best antigen preparation, adjuvants, and delivery routes before considering CS21 phase I safety and immunogenicity clinical studies.

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Highlights

- Genetic diversity found among LngA predicted epitopes from worldwide ETEC strains
- CS21 and LngA are highly immunogenic in a murine vaccine model
- Anti-LngA antibodies cross-react with genetically diverse CS21 and CS8 pili
- CS21 and LngA immunization inhibit ETEC colonization of mice intestine.

Figure 1. Protein sequence alignment of 19 LngA variants (A) and type IV pili major subunits LngA, CofA, BfpA, and TcpA (B). Two major genetic variation regions in LngA were shown in the red lines, which span from residues 56 to 77 and 148 to 197. Two disulfide-bond-forming cysteines in the major subunits of type-4 pili are indicated by red arrows. Five potential surface accessible B cell epitopes were identified at residues ⁵⁰AYQRDGKYPDFV⁶¹, ⁷⁰TIKTDTSIGP⁷⁹, ⁸⁹ITPDEVRNN⁹⁷, ¹⁰⁹LTSNGAQV K¹¹⁷, and ¹⁷⁸GNNGQTTLT¹⁸⁶, tentatively designated as Epitope 1 to 5 (EP1 to EP5).

Figure 2.

The potential linear B cell epitopes of LngA major subunit derived from strain E9034A predicted by Bepipred Linear Epitope Prediction (A), Parker Hydrophilicity Prediction (B), and Emini Surface Accessibility Prediction (C) of Antibody Epitope Prediction on IEDB Analysis Resources (<http://tools.immuneepitope.org/bcell/>). Five potential B cell epitopes are tentatively designated as EP1-5 in red boxes.

Figure 3. LngA-recombinant and CS21 pili antigens recognized by monoclonal anti-LngA and polyclonal murine sera

Panel A. LngA-his tag recombinant and CS21 purified native pili preparation were separated in an SDS-PAGE gel. The Coomassie blue stained gel is shown on the left and the Western blot labeled with anti-LngA monoclonal antibody is shown on the right. CS21 pili preparations were derived from E9034A ETEC strain; LngA-his tag protein was obtained from Genscript; BL21 *E. coli* was used as negative control; and E9034A *lngA* pili preparation was used as LngA negative control. **Panel B.** Western blot of CS21 and CS8 pili from ETEC strains from different geographic locations using murine anti-LngA polyclonal sera. Purified CS21 and CS8 pili from ETEC strains originated in different countries were evaluated by Western blot using two different antisera, one derived from a mouse immunized IP with LngA-his tag antigen plus IFA and the second obtained from a mouse immunized IP with CS21 pili antigen plus IFA. Pili preparations from *E. coli* DH5 α , *E. coli* BL21 and CS21⁺ ETEC E9034A strains were used as negative and positive controls and LngA-His recombinant protein was used as LngA protein control. The size of LngA is 22 kDa.

Figure 4.

Anti-LngA antibody responses in mice immunized with LngA recombinant protein. Mice were immunized with LngA plus IFA, LngA plus ALUM, or LngA plus CT via intraperitoneal routes three times at a two-week intervals. Controls were mock immunized with PBS+IFA or PBS + ALUM. The anti-LngA serum IgG (A), fecal IgA (B), and intestine wash IgA (C) were titrated by ELISA. The anti-LngA serum IgG responses increased steadily overtime until reaching a peak response at 37 days after the primary immunization (D). The closed circles are the control mice and the open circles are the immunized mice. The asterisk (*) indicates p -value <0.05 .

Figure 5.

Anti-LngA and anti-CT antibody responses in mice immunized with CS21 pili antigen and CT adjuvant. Mice were immunized with CS21 plus ALUM via subcutaneous injection (SC), CS21 plus CT via intranasal route (IN), CS21 plus IFA via intraperitoneal route (IP), or CS21 plus ALUM via IP route three times at a two-week intervals. Controls were mock immunized with PBS plus corresponding adjuvants. Naïve mice were not immunized nor challenged. The anti-LngA serum IgG (A), fecal IgA (B), intestine wash IgA (C) and anti-CT antibody (D) were titrated by ELISA. The closed circles are the control mice and the open circles are the immunized mice. The asterisk indicates p -values < 0.05 .

Figure 6.

Effect of LngA and CS21 immunization on gut colonization by measurement of bacterial shedding from mouse feces. To evaluate the role of CS21 and LngA in protection against gut colonization by ETEC, bacterial shedding was measured from mice feces collected daily by quantification of ETEC colony-forming units per gram of feces. Panel A. Mice immunized with LngA + IFA or LngA + CT via intraperitoneal (IP) route. Panel B. CS21 + CT via the intranasal route (IN). Panel C. CS21+ ALUM via the subcutaneous route (SC). Panel D, CS21+ IFA via IP route. Panel E, CS21 + ALUM via IP route. All mice except the naïve group were orally challenged with CS21⁺ ETEC E9034A strain. Mice feces were collected and suspended in sterile PBS and the bacteria CFU per gram of feces was determined by plating feces suspension on MacConkey plates. The asterisk (*) indicates a p -value <0.05.

Table 1

Strains and their characteristics in this study

Strains	Serotype	Toxin	Pili	LngA alleles	Phylogenetic groups	Countries	References
DH5a	N.K.	None	None	N.A.	N.K.	N.K.	Laboratory strain
E9034A/ pCM117	O8:H9	LT, ST	CS21, CS3	Group 1	A	N.A.	This study
E9034A <i>IngA</i>	O8:H9	LT, ST	CS21, CS3	Group 1	A	Caribbean	Clavijo et al. (2010)
E9034A	O8:H9	LT, ST	CS21, CS3	Group 1	A	Caribbean	Levine et al. (1984)
10159-a	N.K.	LT	CS21,	Group 2	B1	Chile	Beatty et al. (2006)
COC1129	O128:H45	ST	CS21, CFA/I	N.K.	N.K.	Colombia	Gomez-Duarte et al. (2013)
COC40	O25:H16	LT	CS21, CS6	N.K.	N.K.	Colombia	Gomez-Duarte et al. (2013)
M452-C1	O20:H-	ST	CS21, CFA/I	Group 2	A	Morocco	Blank et al. (2000)
B2C	O6:H16	LT, ST	CS21, CS2, CS3	Group 1	A	Vietnam	Adachi et al. (2002)
B7A	O148:H28	LT, ST	CS21, CS6	Group 2	B1	Vietnam	Adachi et al. (2002)
M403-C3	N.K.	N.K.	CS8	N.A.	A	Bangladesh	Gomez-Duarte et al. (2007)
E2528C1	O25:NM	LT	CS8, CS14	N.A.	A	Caribbean	Adachi et al. (2002)
WS6866B-2	N.K.	LT	CS8	N.A.	N.K.	Egypt	Guerra et al. (2014)

CS= coli surface antigen, CFA/I= colonization factor antigen I, NM=nonmotile, N.A.=not applicable, N.K.=not known, LT=heat-labile enterotoxin, ST=heat-stable enterotoxin.