Safety and immunogenicity of intramuscularly administered CS6 subunit vaccine with a modified heat-labile enterotoxin from enterotoxigenic Escherichia coli

Tida Lee a,1, Ramiro L. Gutiérrez a, Milton Maciel a,b,2, Steven Poole a,b,2, Kayla J. Testa a,b, Stefanie Trop a,b,3, Christopher Duplessis a, Alison Lane d,4, Mark S. Riddle d,5, Melinda Hamer c,d,6, Ashley Alcala a,b, Michael Prout a,7, Nicole Mai e, Rahsan Erdem e, A. Louis Bourgeois e, Chad K. Porter a,*

a Enteric Diseases Department, Naval Medical Research Center, United States
b Henry M. Jackson Foundation, United States
c Walter Reed Army Institute of Research, United States
d Uniformed Services University, United States
e PATH, United States

ARTICLE INFO

Article history:
Received 4 May 2021
Received in revised form 5 August 2021
Accepted 9 August 2021
Available online 18 August 2021

Keywords:
Enterotoxigenic Escherichia coli
Double mutant heat-labile enterotoxin, ETEC
dmLT
Vaccine
Intramuscular
CS6
CssBA

ABSTRACT

Introduction: Enterotoxigenic Escherichia coli (ETEC) is a common cause of infectious diarrhea and a leading cause of morbidity and mortality in children living in resource-limited settings. It is also the leading cause of travelers’ diarrhea among civilian and military travelers. Its dual importance in global public health and travel medicine highlights the need for an effective vaccine. ETEC express colonization factors (CFs) that mediate adherence to the small intestine. An epidemiologically prevalent CF is coli surface antigen 6 (CS6). We assessed the safety and immunogenicity of a CS6-targeted candidate vaccine, CssBA, co-administered intramuscularly with the double-mutant heat-labile enterotoxin, dmLT [LT (R192G/L211A)].

Methods: This was an open-label trial. Fifty subjects received three intramuscular injections (Days 1, 22 and 43) of CssBA alone (5 mg), dmLT alone (0.1 mg) or CssBA (5, 15, 45 mg) + dmLT (0.1 and 0.5 mg). Subjects were actively monitored for adverse events for 28 days following the third vaccination. Antibody responses (IgG and IgA) were characterized in the serum and from lymphocyte supernatants (ALS) to CS6 and the native ETEC heat labile enterotoxin, LT.

Results: Across all dose cohorts, the vaccine was safe and well-tolerated with no vaccine-related severe or serious adverse events. Among vaccine-related adverse events, a majority (98%) were mild with 79% being short-lived vaccine site reactions. Robust antibody responses were induced in a dose-dependent manner with a clear dmLT adjuvant effect. Response rates in subjects receiving 45 mg CssBA and 0.5 mg dmLT ranged from 50 to 100% across assays.

Conclusion: This is the first study to demonstrate the safety and immunogenicity of CssBA and/or dmLT administered intramuscularly. Co-administration of the two components induced robust immune responses to CS6 and LT, paving the way for future studies to evaluate the efficacy of this vaccine target and development of a multivalent, subunit ETEC vaccine.

Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

Enterotoxigenic Escherichia coli (ETEC), one of several pathotypes of diarrhoeagenic E. coli, causes a secretory diarrhea that can range in presentation from mild discomfort to cholera-like purging [1,2]. ETEC is one of the most common causes of childhood diarrhea in low-and middle-income countries (LMIC), and the estimated number of ETEC-attributable deaths varies from 23,000
to 42,000 (with large uncertainty intervals) annually among infants and young children [3]. In addition to the morbidity associated with acute diarrhoeal illness, recent data indicate that ETEC infections may also be associated with growth faltering and delayed cognitive development [4,5], further magnifying the pathogen-specific acute and longer-term morbidity and its negative economic impact [6]. ETEC is also the leading cause of travellers’ diarrhoea, implicated in 30–50% of cases [7-9].

While antibiotics have traditionally been effective at clearing ETEC infections, strains have become increasingly resistant [1,10,11]. Enterobacteriaceae are included on the critical World Health Organisation (WHO) pathogen priority list for the development of new antimicrobials [12] and classified as an urgent antimicrobial resistance (AMR) threat by the US Centers for Disease Control and Prevention [13]. Combined, these data highlight the need for primary prevention. This is supported by the recommendations from the Wellcome Trust and the Boston Consulting Group, as well as the WHO to accelerate vaccine development for enteric E. coli, including ETEC [14,15]. In addition, ETEC vaccine development was also recently reaffirmed as a WHO priority [16].

While ETEC vaccine development has made significant advancements over the past several decades, no vaccine is currently available [17]. To fill this important gap in public health and travel medicine, the US Naval Medical Research Center (NMRC) has been advancing a subunit candidate vaccine based on subunits of common colonization factors (CFs), surface-exposed polymorphic protein appendages that mediate initial adhesion and colonization of the small intestine. Antibodies directed to CFs have been shown to be protective in natural and experimental infections [18-24]. One of the most prevalent and epidemiologically important CFs is an atypical polymeric antigen termed CS6 [22,25,26]. CS6 is a heteropolymor composed of two subunits, CssA and CssB, in an approximate 1:1 ratio [27]. CS6 binds to Caco-2, INT407 and HT-29 cells [28-30]. Both purified CS6 and recombinant CssB fused to glutathione-S-transferase (GST) have been shown to bind to intestinal glycosphingolipid sulphatide by thin layer chromatography [31]. CS6-expressing ETEC strains are important causes of diarrhoeal illness among infants and young children in low resource settings and travellers to ETEC-endemic areas [16,32-34]. Consequently, it is considered to be an essential component in most ETEC vaccine candidates currently under development [16,17].

We previously reported on biochemical properties of in cis donor strand complemented variants ofCssA and CssB and characterized the immune responses in mice [30]. Based on multiple lines of evidence, ndt1dsc16bsCssBA (hereafter termedCssBA), a CssB-CssA fusion in which the N-terminal 14 amino acids of CssB have been removed and a heterologous CssB-derived donor strand used to complement the C-terminal CssA, was selected as the lead vaccine prototype. Additionally, intramuscular immunization with a formulation containing the CssBA fusion, constructed with the CssA and CssB alleles from ETEC strain B7A, significantly protected Aotus nancymae non-human primates against diarrhoeal disease after challenge with the CS6-expressing ETEC strain B7A [35]. This product was produced under current Good Manufacturing Practice (cGMP) and as detailed here, evaluated for safety and immunogenicity when administered intramuscularly with the double mutant E. coli heat-labile enterotoxin LT(R192G/L211A) (dmLT) in a Phase 1 clinical trial. The role of dmLT in the vaccine formulation was to induce anti-LT toxin immunity and to adjuvant anti-CssBA responses [36,37]. This Phase 1 trial was also the first clinical study in which dmLT was included in a candidate vaccine given by the intramuscular route.

2. Methods

2.1. Study design

CssBA (a protein fusion of the CssB and CssA subunits of CS6 in which the N-terminal 14 amino acids have been removed from CssB and a sixteen amino acid, heterologous CssB-derived donor strand is used to complement the C-terminal CssA) was selected as the lead vaccine candidate after the biochemical and immunological characterization of a panel of in cis donor strand complemented (dsc) variants of CS6 subunit-derived antigens [30]. The source of the CssA and CssB alleles for constructing CssBA was ETEC strain B7A [35]. The vaccine was manufactured at the Walter Reed Army Institute of Research (WRAIR) Pilot Bioproduction Facility (PBF) (Silver Spring, MD) as lot 1880.

Given the likely importance of anti-LT responses in an ETEC vaccine and due to its adjuvant properties, CssBA was co-administered with dmLT (manufacturer: IDT Biologika Corporation; Rockville, MD: Lot: 001 08 16). Currently, there is no previous published human experience of dmLT given by the intramuscular route; however, several first in human studies have been initiated evaluating dmLT intradermally (NCT02531685, NCT01644565).

This study was designed as an open-label, Phase 1 clinical trial in which a total of 50 subjects were scheduled to receive three intramuscular (IM) injections of either CssBA alone, dmLT alone or CssBA + dmLT, with the combined products then dose-escalated by cohort. The vaccine was administered to alternating deltoid regions on Days 1, 22, and 43, and each subject received the same dose at each vaccination dependent upon cohort assignment. Cohort A was considered a pilot group where five subjects received all three vaccinations with either 5 μg CssBA (A-1) or 0.1 μg dmLT (A-2). Subjects in Cohort A were monitored for safety seven days after the third vaccination, before enrollment of subjects in Cohort B. Cohort B consisted of ten subjects receiving 5 μg CssBA dose co-administered with 0.1 μg of dmLT. Following completion of the 3-dose series, the dmLT dose increased to 0.5 μg and co-administered with 5 μg CssBA in Cohort C. The dose of CssBA was then scheduled to be increased to 15 μg (Cohort D) and 45 μg (Cohort E) in subsequent cohorts with either 0.1 or 0.5 μg of dmLT dependent on the safety profile observed with co-administration with 5 μg CssBA. Given no safety concerns in cohort C, subsequent cohorts D and E received the 0.5 μg dmLT dose. Each subject received three doses and all subjects within a cohort were enrolled on the same day.

Healthy adult (aged 18–45 years) male and non-pregnant female subjects were recruited from the greater Washington, DC area through the use of multiple IRB-approved media advertising formats. Interested subjects contacted the WRAIR Clinical Trials Center and discussed details of the trial with a member of the recruitment staff, following which an appointment for briefing/screening was arranged. Subjects were enrolled following completion of an informed consent process which included a taped or in-person presentation about the study, passing a comprehension test, a one-on-one discussion with a clinical investigator and signing the informed consent document. Subjects with any of the following were excluded: significant acute or chronic diseases, immunosuppressive disorders or medication, regular use of anti-diarrhoeal, anti-constipation, or antacid therapy, an abnormal stool pattern (<3 stools per week, or >3 stools per day), participating in other investigational product research within 30 days before the planned date of first vaccination or anytime through the last study safety visit, positive blood test for Hepatitis B surface antigen, Hepatitis C virus, or Human Immunodeficiency Virus-1/2, or clinically significant abnormalities on basic laboratory screening.
Subjects with abnormal skin history or findings potentially affecting local adverse event assessments were also excluded. To increase the likelihood of vaccinating immunologically naïve subjects, only those with no history (in the past three years) of ETEC or cholera exposure and with no travel (in the past three years) to countries where those pathogens are endemic were eligible.

The two components were co-formulated for administration immediately before IM vaccination. The vaccine (0.25 ml) was delivered to alternating deltoids using a 23 gauge one-inch needle. Subjects were observed in the clinic for at least 30 min post-vaccination and vital signs were collected. Memory aids were provided to subjects to facilitate adverse event reporting. Subjects returned for follow-up one and seven days after the first vaccination for a clinical evaluation which included vital signs, adverse event assessment, and review of changes in medical history, concomitant medications, and targeted clinical assessment. For subsequent vaccinations, each subject underwent these same clinical evaluations at Days 2 and 8 post-vaccination. Clinical investigators reviewed the memory aid with subjects at each clinical visit. Approximately 6 and 12 months from the last vaccination subjects were contacted by telephone to assess for a final safety assessment.

The decision to advance to the next cohort was based solely on the safety assessment. A dose level with no occurrence of stopping criteria prompted moving to the next higher level. All safety data were summarized and reviewed with the Safety Review Committee (SRC) prior to advancing to dose escalation. Approximately one week after Cohort A (A-1 and A-2) received the third vaccination dose (Day 50), an interim Safety Report was prepared by the PI and Study Statistician for review by the SRC. The content of the report included all adverse events as well as relevant safety endpoints. The SRC’s concurrence to advance to the next cohort was made and provided in written format.

2.2. Safety assessment

Safety monitoring was undertaken using in-person symptom surveillance, symptom memory aids, and targeted physical exams. Adverse event (AE) monitoring surveyed and specifically inquired about fever (oral temperature > 100.4°F), malaise, headache, rash, pain, and extremity pain, swelling, or local reactions. Clinical definitions were used to grade the severity of symptoms as mild (not interfering with routine activities), moderate (interfering with but not precluding routine activities) and severe (preventing routine activities). Blood for complete blood counts and serum chemistry was collected throughout the vaccination phase.

2.3. Immunological assessments

Immunogenicity measures were assessed throughout the study. Serum samples were assayed for anti-CS6 and –LT antibody (Ab) IgG and IgA titers by enzyme-linked immunosorbent assay (ELISA). Antigen (Ag)-specific IgG assays were performed on Nunc™ MicroWell™, while IgA assays were performed on Nunc™ MicroWell™ Maxisorb™ (Thermo Scientific, Rochester, NY) 96-well plates. For anti-CS6-specific assays, plates were coated with CS6 (BEI, NIH/C176) or 1% Casein (Sigma-Aldrich) for LT assays, or 0.5 µg/mL peroxidase-conjugated goat anti-human IgG (KPL, Gaithersburg, MD) for LT IgG assays, or 0.5 µg/mL peroxidase-conjugated goat anti-human IgG (KPL, Gaithersburg, MD) for LT IgG assays, or 1.5 h at 37 °C in a humidified chamber. Plates were washed 5 times with PBS-T followed by addition of 0.25 µg/mL biotin-conjugated anti-human IgG or IgA (KPL), for CS6 IgG and IgA, and LT IgA assays, or 0.5 µg/mL peroxidase-conjugated goat anti-human IgG (KPL, Gaithersburg, MD) for LT IgG assays, for 1.5 h at room temperature (RT). After further washes, assays performed with biotin-conjugates received ExtrAvidin®-Peroxidase (Sigma-Aldrich) at 1:2000 for 30 min at RT. After final washes, 2,2’-azin o-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS; KPL) substrate was used to develop assays based on peroxidase, while 3,3’,5,5-tetramethylbenzidine (Ultra-TMB; Thermo Scientific) substrate was employed to develop assays based on biotin-streptavidin, according to the manufacturer recommendations. After a 20–30 min incubation, optical density (OD) was measured at 450 or 405 nm for ABTS or Ultra-TMB, respectively, using a Multiskan™ ELISA reader with Ascent™ software (Thermo Scientific), which calculated the final antibody titers. The cut-off for each plate was calculated by the average of the background wells OD plus a fixed value of 0.4. A linear regression was fitted to the experimental data and the endpoint titer was determined as the reciprocal of the interpolated sample dilution that intersected with the cut-off and then log10-transformed. All pre- and post-vaccination samples from a given subject were assayed concurrently on the same plate and each sample was tested in duplicate. The average log10 reciprocal titer for the duplicate tests was calculated as the final result. Serum samples with OD below the cut-off, even at the top serum dilution, were assigned a value of one-half of the lower detection limit for analysis.

After isolation by a Ficoll gradient with Cell Preparation Tubes with sodium heparin (CPT™, Becton, Dickson and Company, Franklin Lakes, NJ, US), peripheral blood mononuclear cells were cultured at 5x10^6 cells/mL in 24-well plates (Becton, Dickson and Company, Falcon™) in compete RPMI (10% FCS, 1% Penicillin/Streptomycin, 1% GlutMax) without stimulation for 72 h. Once collected, supernatants were kept at −80 °C until assayed by ELISA for anti-CS6 and anti-LT IgG and IgA antibody as described above, except for the initial sample dilution, which were performed as follows: 1:8 for anti-CS6 IgG, 1:5 for anti-CS6 IgA, and 1:5 for anti-LT IgG and IgA. A positive response for serology and antibody from lymphocyte supernatant (ALS) was defined as a four-fold rise in antibody titers between pre- and post-vaccination samples.

2.4. Statistical analysis

Given the early stage of the product concept/testing, the sample size for this study was designed to evaluate preliminary safety data but not designed to show statistically significant differences between cohorts. Nominal data (proportion with adverse events, proportion meeting immunologic responder definitions) were analyzed by Pearson’s Chi-square test (or Fisher’s exact test) to compare dose levels. For immune responses, antibody titers were log10-transformed for analysis. Between cohorts, comparisons were examined using analysis of variance (ANOVA) and over time using repeated measures ANOVA. Only subjects who received at least two vaccine doses were included in immunologic analysis. All analyses were performed using SAS v 9.4 (Cary, NC) and GraphPad Prism 7.0 for Mac (GraphPad Software, La Jolla, CA) and a two-sided alpha = 0.05.
3. Results

3.1. Safety

A total of 97 subjects were screened to identify 50 eligible subjects (Fig. 1). The most common reasons for ineligibility were schedule conflict (12/43), medical history (10/43), and history of travel to an ETEC endemic area (9/43). Among the 54 eligible subjects, 50 were ultimately enrolled in the study with a median age of 30 years (interquartile range, IQR: 26–37). Of the included population, 60% were female, 46% were black and 48% were white, and the majority were non-Hispanic (Table 1). Five subjects were withdrawn from the study, two subjects after one dose, two subjects after two doses, and one subject was withdrawn after three doses. None of the subject withdrawals were due to AEs but were due to either an inability to comply with the study procedures or starting medication that precluded the subjects’ continued eligibility.

Across all study cohorts, the vaccine was safe and well-tolerated. The frequency of vaccine-related systemic AEs differed slightly across cohorts, with the lowest frequency in subjects receiving CssBA alone (Table 2). Loose or soft stools and headache at least possibly related to the vaccine occurred in less than 20% of subjects in all cohorts. Transient myalgia was observed in 10.0–70.0%; D: 80.0%; E: 80.0%). Vaccine site pain was more prevalent among subjects receiving CssBA alone or 0.1 μg dmLT; however, this difference was not statistically significant (p = 0.5).

Adverse events at the vaccine site were mostly mild (98%) with erythema and tenderness being the most common reactions across cohorts (Table 3). Erythema occurred in the majority of subjects receiving dmLT (70% of all subjects; A: 80.0%; B: 70.0%; C: 70.0%; D: 80.0%; E: 80.0%). Vaccine site pain was more prevalent among subjects in Cohorts C (50.0%), D (80.0%), and E (80.0%) compared to Cohorts A (10.0%) and B (20.0%) (Fisher’s exact, p = 0.003). Additionally, induration was only observed in subjects receiving the 0.5 μg of dmLT (p = 0.002) and there appeared to be a CssBA-driven dose response effect on the timing of the induration. Specifically, 6 (66.7%) subjects in Cohort C (5 μg CssBA, 0.5 μg dmLT) experienced induration only after the third dose. In Cohorts D (15 μg CssBA, 0.5 μg dmLT) and E (45 μg CssBA, 0.5 μg dmLT), induration increased with each dose (Cohort D- dose 1: 0.0%, dose 2: 30.0%, dose 3: 50.0%; Cohort E- dose 1: 20.0%, dose 2: 40.0%, dose 3: 66.7%). Erythema and pain were seen more frequently after the third dose among subjects in all cohorts (p = 0.013 and p = 0.002, respectively). In Cohort D, 2 (20.0%) and 6 (60.0%) subjects had pruritus after the second vaccination and third vaccinations, respectively, and only two subjects (one each in Cohorts B and C) had pruritus (p < 0.0001).

3.2. Immunogenicity

The frequency of anti-CS6 serologic responses in subjects receiving 5 μg CssBA alone (Group A-1) was low across all assays (Table 4). When 5 μg CssBA was co-administered with 0.1 μg of dmLT (Group B), the anti-CS6 IgA response rate remained low; however, serum IgG responses increased to 77.8% (7/9). Increasing the dmLT dose to 0.5 μg (Group C) induced a higher proportion of serum IgG and IgA responders (100% (9/9) and 44.4% (4/9), respectively). Anti-CS6 serum IgG and IgA antibody levels increased over the study period with peak titers observed on Day 71 for subjects receiving CssBA + dmLT (Fig. 2A and B). Anti-CS6 IgG titers were significantly higher in subjects receiving 5 μg CssBA + 0.1 μg dmLT compared to those receiving 5 μg CssBA alone (p = 0.0001). Increasing the dmLT dose to 0.5 μg dmLT in the subsequent cohorts (C, D and E) further increased the CS6 IgG titers in comparison to cohort A-1 (p < 0.0001) (Fig. 2A). A significant increase in peak anti-CS6 IgA antibody titers was also observed between groups B and C when the dmLT dose was increased from 0.1 to 0.5 μg (p = 0.04, Fig. 2B). Anti-CS6 IgA response rate increased with the addition of 0.1 μg dmLT (IgG: 60% (3/5) to 100% (8/8); IgA: 0% (0/5) to 33% (3/9)) (Table 4). Increasing the dose of dmLT to 0.5 μg induced >80% ALS response rates in all subsequent groups regardless of CssBA dose. Overall, the ALS titers peaked after the third immunization, on Day 50 (Fig. 3A and B). Among all groups, vaccination with 5 μg CssBA + 0.5 μg dmLT (Group C) elicited the highest mean titers; however, those titers were not significantly different than those observed in subjects receiving higher doses of CssBA with 0.5 μg dmLT.

Anti-LT serum antibody responses were frequent with significant increases over baseline titers for all groups receiving dmLT (Table 4; Fig. 2C and 2D). Serum anti-LT IgG antibody titers appeared to increase over the vaccination series (Fig. 2C), while anti-LT IgA titers peaked after first or second vaccination, depending on the group (Fig. 2D). All groups receiving dmLT had a significant increase in ALS anti-LT IgG and IgA levels (Table 4; Fig. 3C and D). ALS anti-LT IgG titers increased significantly after the first vaccination for Groups C, D, and E (p < 0.05 for all comparisons) and continued to increase significantly after the second vaccination in Groups C and E (p < 0.01 and p < 0.001, respectively) (Fig. 3C). By Day 50, seven days after the third vaccination, all groups had comparable ALS anti-LT IgG titers. Compared to baseline, ALS anti-LT IgA antibody titers only increased significantly in group D after the first vaccination, by Day 8 (p < 0.05, Fig. 3D). Significant increases in ALS anti-LT IgA levels were also seen in Group E after the second vaccination, by Day 29 (p < 0.05).

Strong correlations were observed between serological and ALS responses for anti-CS6 IgG, and anti-LT IgG and IgA antibodies (all Spearman r = 0.72 to 0.75, p < 0.0001; Suppl. Fig. 1A, C and D), while a modest correlation was observed for the anti-CS6 IgA antibody response (Spearman r = 0.32, p < 0.001; Suppl. Fig. 1B).

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2021.08.032.

4. Discussion

This is the first publication on the safety and immunogenicity of the CssBA subunit candidate vaccine and the first evaluation of dmLT administered IM. The investigational products were well-tolerated at all doses and the majority (97.0%) of vaccine-related AEs were considered mild. The most common symptom related to vaccination was erythema at the site of administration, observed in 70% of vaccine recipients. Vaccination site pain was also common (48%) and local site reactions seemed most prevalent in subjects receiving dmLT at the 0.5 μg dose. Others have evaluated dmLT alone or as an adjuvant through oral, sublingual, and intradermal (ID) routes, without safety concerns [38,39] (NCT02531685). For the ID route, the highest dose of dmLT tested (0.5 μg) induced >80% ALS response rates in all subsequent groups regardless of CssBA dose. Overall, the ALS titers peaked after the third immunization, on Day 50 (Fig. 3A and B). Among all groups, vaccination with 5 μg CssBA + 0.5 μg dmLT (Group C) elicited the highest mean titers; however, those titers were not significantly different than those observed in subjects receiving higher doses of CssBA with 0.5 μg dmLT.
Fig. 1. Study population diagram. a. Subject withdrawn after first dose due to a schedule change leading to inability to attend future visits; b. subject withdrawn after two doses due to schedule changes that prevented the subject from attending future visits; c. one subject received three doses but was withdrawn after the third dose due to behavioral issues; d. Subject withdrawn after first dose due to initiation of a medication that precluded eligibility; e. Subject was withdrawn after two doses due to initiation of a medication that precluded eligibility.

Table 1
Demographics of study population.

|        | A-1 (N = 5) | A-2 (N = 5) | B (N = 10) | C (N = 10) | D (N = 10) | E (N = 10) | Total (N = 50) |
|--------|-------------|-------------|------------|------------|------------|------------|----------------|
| N      | 5           | 5           | 10         | 10         | 10         | 10         | 50             |
| Median Age (1Q, 3Q) |
| Male   | 32 (30, 41) | 29 (27, 37) | 29.5 (26, 32) | 31.5 (27, 37) | 28 (22, 38) | 29.5 (25, 33) | 30 (26, 37) |
| Female | 4 (80.0)    | 2 (40.0)    | 5 (50.0)   | 3 (30.0)   | 5 (50.0)   | 7 (70.0)   | 20 (40.0)      |
| Race   |
| Asian  | 0 (0.0)     | 0 (0.0)     | 0 (0.0)    | 0 (0.0)    | 0 (0.0)    | 0 (0.0)    | 2 (4.0)        |
| Black  | 4 (80.0)    | 4 (80.0)    | 3 (30.0)   | 5 (50.0)   | 3 (30.0)   | 4 (40.0)   | 23 (46.0)      |
| White  | 1 (20.0)    | 1 (20.0)    | 7 (70.0)   | 7 (70.0)   | 5 (50.0)   | 7 (70.0)   | 30 (60.0)      |
| Multi-racial | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (10.0) | 1 (2.0) |
| Ethnicity |
| Non-Hispanic/Latino | 5 (100.0) | 3 (60.0) | 9 (90.0) | 10 (100.0) | 8 (80.0) | 9 (90.0) | 44 (88.0) |
| Hispanic/Latino | 0 (0.0) | 2 (40.0) | 1 (10.0) | 0 (0.0) | 2 (20.0) | 1 (10.0) | 6 (12.0) |

Table 2
Systemic adverse events considered to be at least possibly related to the vaccine.

| Adverse Event           | Cohort A-1 (N = 5) | Cohort A-2 (N = 5) | Cohort B (N = 10) | Cohort C (N = 10) | Cohort D (N = 10) | Cohort E (N = 10) | Total (N = 50) |
|-------------------------|---------------------|---------------------|------------------|------------------|------------------|------------------|----------------|
| Abdominal Pain          | 0 (0.0)             | 0 (0.0)             | 0 (0.0)          | 0 (0.0)          | 1 (10.0)         | 0 (0.0)          | 1 (2.0)        |
| Arthralgia              | 0 (0.0)             | 0 (0.0)             | 0 (0.0)          | 0 (0.0)          | 1 (10.0)         | 1 (10.0)         | 2 (4.0)        |
| Aspartate Aminotransferase Increased | 0 (0.0) | 1 (20.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (2.0) |
| Chills                  | 0 (0.0)             | 0 (0.0)             | 0 (0.0)          | 0 (0.0)          | 1 (10.0)         | 0 (0.0)          | 1 (2.0)        |
| Loose/Soft stools       | 0 (0.0)             | 1 (20.0)            | 2 (20.0)         | 2 (20.0)         | 2 (20.0)         | 1 (10.0)         | 8 (16.0)       |
| Fatigue                 | 0 (0.0)             | 0 (0.0)             | 1 (10.0)         | 0 (0.0)          | 0 (0.0)          | 0 (0.0)          | 1 (2.0)        |
| Headache                | 1 (20.0)            | 0 (0.0)             | 1 (10.0)         | 1 (10.0)         | 2 (20.0)         | 0 (0.0)          | 5 (10.0)       |
| Leukopenia              | 0 (0.0)             | 0 (0.0)             | 0 (0.0)          | 0 (0.0)          | 1 (10.0)         | 0 (0.0)          | 1 (2.0)        |
| Malaise                 | 0 (0.0)             | 0 (0.0)             | 0 (0.0)          | 0 (0.0)          | 2 (20.0)         | 1 (10.0)         | 3 (6.0)        |
| Myalgia                 | 0 (0.0)             | 0 (0.0)             | 0 (0.0)          | 1 (10.0)         | 2 (20.0)         | 2 (20.0)         | 5 (10.0)       |
| Nausea                  | 0 (0.0)             | 0 (0.0)             | 0 (0.0)          | 1 (10.0)         | 1 (10.0)         | 0 (0.0)          | 2 (4.0)        |
| Pruritus, inner arm     | 0 (0.0)             | 0 (0.0)             | 0 (0.0)          | 0 (0.0)          | 0 (0.0)          | 1 (10.0)         | 1 (2.0)        |
| Pyrexia                 | 0 (0.0)             | 0 (0.0)             | 0 (0.0)          | 0 (0.0)          | 1 (10.0)         | 0 (0.0)          | 1 (2.0)        |
Proportion of Subjects Demonstrating an Immunologic Response (Serology, ALS) to Immunizing Antigens.

Vaccine-site reactions by dose.

m 0.5-g vaccination induced robust activation of the B cell compartment against CS6 and LT in a dose-dependent manner, with the most robust responders observed in groups receiving CssBA with the 0.5 µg dmLT dose. Together, these data suggest that recombinant CssBA co-administered IM with a potent adjuvant such as dmLT is able to induce a robust immune response against the native antigen CS6. Additionally, dmLT also induced a robust anti-LT response. While anti-LT titers significantly increased following the first vaccination among subjects receiving the 0.5 µg dmLT dose, overall anti-CS6 responses tended to increase in magnitude and frequency with the dmLT dose.

Adverse event severity was characterized as mild: not interfering with routine activities; moderate (mod): interfering with but not precluding routine activities; severe (sev): preventing routine activities.

Two subjects (1 in Cohort B and 1 in Cohort C) had bruising at the vaccination site.

Responder defined as ≥ 4-fold rise in baseline reciprocal endpoint titer.

One subject excluded because the subject only had one sample post vaccination despite receiving two vaccinations.

One subject excluded because the subject only had one sample post vaccination despite receiving two vaccinations.

Table 3
Vaccine-site reactions by dose.

Table 4
Proportion of Subjects Demonstrating an Immunologic Response (Serology, ALS) to Immunizing Antigens.

Coli Surface Antigen 6 (CS6)

Heat labile toxin (LT)

Serology (IgG)

Serology (IgA)
also be co-administered with dmLT. Preclinical investigations of the multivalent formulation in small rodents demonstrated that each vaccine component elicited functional antibodies against its respective CF (M. Maciel, personal communication). Data indicate that this quadrivalent vaccine would provide coverage of the majority of ETEC strains causing disease in adult travelers and children living in LMICs [32,33].

This subunit-based vaccine approach is amenable to the characteristics delineated in the WHO’s recently published draft preferred product characteristics for an ETEC vaccine [16].
Additionally, given that this vaccine is administered intramuscu-
larly, it may offer an advantage over orally administered vaccines
which have traditionally been met with reduced immunogenicity
when administered to infants and young children in endemic set-
tings [43–45]. Furthermore, its ability to be combined with other
enteric vaccines under development, including *Shigella* and
typhoid, may increase the value of the ETEC subunits vaccine
approach [16,43,44,46].

5. Disclaimer

The views expressed in this article are those of the authors and
do not necessarily reflect the official policy or position of the
Department of the Navy, Department of the Army, Department of
Defense, nor the U.S. Government. This is a US Government work.
There are no restrictions on its use. There were no financial con-
flicts of interests among any of the authors.

6. Copyright statement

Some authors are employees of the U.S. Government. This work
was prepared as part of their official duties. Title 17 U.S.C. §105
provides that “Copyright protection under this title is not available
for any work of the United States Government.” Title 17 U.S.C. §101
defines a U.S. Government work as a work prepared by a military
service member or employee of the U.S. Government as part of that
person’s official duties.

**Funding**

This study was funded by PATH under a Cooperative Research
and Development Agreement (NMR 9589). This work was sup-
pported, in part, by the Bill & Melinda Gates Foundation
(OPT112376). Under the grant conditions of the Foundation, a
Creative Commons Attribution 4.0 Generic License has already
been assigned to the Author Accepted Manuscript version that
might arise from this submission.

**Declaration of Competing Interest**

The authors declare that they have no known competing finan-
cial interests or personal relationships that could have appeared
to influence the work reported in this paper.

**Acknowledgements**

The authors are in debt to Amanda Vazquez and Susan Cicatelli
for their efforts in coordinating the conduct of the trial and to
Aaron Kim, Zuzana Villar and Elizabeth Ward for their efforts to
process blood samples and to perform the immunologic assays
reported here. We would also like to thank the study volunteers
without whom this trial would not have been possible.

**Human volunteers protection**

This study was approved by the ethical review committee of the
Naval Medical Research Center, Silver Spring, MD (NMRC.2016.0013) in compliance with all Federal regulations gov-
erning the protection of human volunteers and registered on Clin-
icalTrials.gov (NCT03404674).

Reference

[1] Qadri F, Svennerholm A-M, Faruque ASC, Sack RB. Enterotoxigenic Escherichia
coli in developing countries: epidemiology, microbiology, clinical features, treatment,
and prevention. Curr Microbiol Rev 2005;18(3):405–83.
[2] Qadri F, Khan AI, Faruque ASG, Begum YA, Chowdhury F, Nair GB, et al.
Enterotoxigenic Escherichia coli and Vibrio cholerae diarrhea, Bangladesh, 2004.
Emerg Infect Dis 2005;11(7):1104–7.
[3] Husangadi D, Smith PG, Gierson R. Considerations for using ETEC and
Shigella disease burden estimates to guide vaccine development strategy.
Vaccine 2017.
[4] Black RE, Brown KH, Becker S. Effects of diarrhea associated with specific
teratopathogens on the growth of children in rural Bangladesh. Pediatrics
1984;73:799–805.
[5] Guerraani D et al. Association of early childhood diarrhea and crypto-
pseudopsis with impaired physical fitness and cognitive function four–seven years later in
a poor urban community in northeast Brazil. Am J Trop Med Hyg 1999;61
(5):707–13.
[6] Bloom DE, Canning D. Policy forum: public health. The health and wealth of
nations. Science 2000;287(5456):1265–9.
[7] Shah N, DuPont HL, Ramsey DJ. Global etiology of travelers’ diarrhea:
systematic review from 1973 to the present. Am J Trop Med Hyg 2009;80
(4):609–14.
[8] Porter CK, Olson S, Hall A, Riddle MS. Travelers’ Diarrhea: An Update on the
Incidence, Etiology, and Risk in Military Deployments and Similar Travel
Populations. Mil Med 2017;182(52):4–10.
[9] Olson S et al. Travelers’ diarrhea: update on the incidence, etiology and risk in
military and similar populations – 1990–2005 versus 2005–2015, does a decade make a difference? Trop Dis Travel Med Vaccines 2019;5:1.
[10] Wennersas C, Erling V. Prevalence of enterotoxigenic Escherichia coli-
associated diarrhea and carrier state in the developing world. J Health Popul
Nutr 2004;22(6):370–92.
[11] Trumble DR. Antibiotic Therapy for Acute Watery Diarrhea and Dysentery.
Mil Med 2017;182(52):17–25.
[12] Taconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, et al.
Discovery, research, and development of new antibiotics: the WHO priority list of
antibiotic-resistant bacteria and tuberculosis. Lancet Infect Dis 2018;18
(3):318–27.
[13] Center for Disease Control and Prevention. Antibiotic Resistance Threats in
the United States; 2019. https://www.cdc.gov/drugresistance/biggest-threats.
html#CDC_AA_refVal=https%3A%2F%2Fwww.cdc.gov%2Fdrugresistance%c2%
2FBiggest_threats.html.
[14] The Boston Consulting Group. Vaccines to tackle drug resistant infections:
An evaluation of R&D opportunities; 2018. https://vaccinesforamr.org/wp-
content/uploads/2018/09/Vaccines_for_AMR.pdf.
[15] World Health Organization. Leveraging Vaccines to Reduce Antibiotic Use
and Prevent Antimicrobial Resistance. https://www.who.int/publications/m/item/
leveraging-vaccines-to-reduce-antibiotic-use-and-prevent-antimicrobial-
resistance [2020 3/31/2021].
[16] World Health Organization. DRAFT WHO Preferred Product Characteristics
for Vaccines against Enterotoxigenic Escherichia coli; 2020. https://www.who.int/
immunization/research/opc-tpp/PPC_ETEC_April_2020_Public_Consultation.
pdf[accessed 1/21/2021].
[17] Zhang W, Sack DA. Progress and hurdles in the development of vaccines
against enterotoxigenic Escherichia coli in humans. Expert Rev Vaccines
2012;11(6):677–94.
[18] Clemens JD, Sack DA, Harris JR, Chakraborty J, Neogy PK, Stanton B, et al.
Cross-protection by B subunit-whole cell cholera vaccine against diarrhea associated
with heat-labile toxin-producing enterotoxigenic Escherichia coli: results of a
large-scale field trial. J Infect Dis 1988;158(2):372–7.
[19] Cravioto A, Reyes RE, Ortega R, Fernández G, Hernández R, López D.
Prospective study of diarrhoeal disease in a cohort of rural Mexican
children: incidence and isolated pathogens during the first two years of life.
Epidemiol Infect 1988;101(1):123–34.
[20] Levine MM, Nalin DR, Hoover DL, Bergquist EJ, Horswick BY, Young CR. Immunity
to enterotoxigenic Escherichia coli. Infect Immun 1979;23(3):729–36.
[21] López-Vidal Y et al. Enterotoxins and adhesins of enterotoxigenic Escherichia
coli: are they risk factors for acute diarrhea in the community? J Infect Dis
1990;162(2):442–7.
[22] Qadri F, Das SK, Faruque ASC, Fuchs GJ, Albert MJ, Sack RB, et al. Prevalence of
toxin types and colonization factors in enterotoxigenic Escherichia coli
isolated during a 2-year period from diarrheal patients in Bangladesh. J Clin
Microbiol 2000;38(1):27–31.
[23] Freedman D, Tacket C, Delehanthy A, Maneval D, Nataro J, Crabb J. Milk
immunoglobulin with specific activity against purified colonization factor
antigens can protect against oral challenge with enterotoxigenic Escherichia coli.
J Infect Dis 1998;177(3):1662–7.
[24] Tacket CO, Losonsky G, Link H, Hoang V, Guerry P, Hilpert H, et al. Protection
by milk immunoglobulin concentrate against oral challenge with
enterotoxigenic Escherichia coli. N Engl J Med 1988;318(19):1240–3.
[25] Jiang Z-D, Lowe B, Verenkar MP, Ashley D, Steffen R, Tornieporth N, et al.
Prevalence of enteric pathogens among international travelers with diarrhea
acquired in Kenya (Mombasa), India (Goa), or Jamaica (Montego Bay). J Infect
Dis 2002;185(4):497–502.
