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RESEARCH PAPER - INVITED

Oral delivery of Hyperimmune bovine serum antibodies against CS6-expressing enterotoxigenic *Escherichia coli* as a prophylactic against diarrhea

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ABSTRACT

Background. Oral administration of bovine antibodies active against enterotoxigenic *Escherichia coli* (ETEC) have demonstrated safety and efficacy against diarrhea in human challenge trials. The efficacy of bovine serum immunoglobulins (BSIgG) against recombinant colonization factor CS6 or whole cell ETEC strain B7A was assessed against challenge with the CS6-expressing B7A.

Methods. This was a randomized, double-blind, placebo-controlled trial in which healthy adults received oral hyperimmune BSIgG anti-CS6, anti-B7A whole cell killed or non-hyperimmune BSIgG (placebo) in a 1:1:1 ratio then challenged with ETEC B7A. Two days pre-challenge, volunteers began a thrice daily, seven day course of immunoprophylaxis. On day 3, subjects received 1×10^{10} CFUs of B7A. Subjects were observed for safety and the primary endpoint of moderate-severe diarrhea (MSD).

Results. A total of 59 volunteers received product and underwent ETEC challenge. The BSIgG products were well-tolerated across all subjects. Upon challenge, 14/20 (70%) placebo recipients developed MSD, compared to 12/19 (63%; $p = .74$) receiving anti-CS6 BSIgG and 7/20 (35%; $p = .06$) receiving anti-B7A BSIgG. Immune responses to the ETEC infection were modest across all groups.

Conclusions. Bovine-derived serum antibodies appear safe and well tolerated. Antibodies derived from cattle immunized with whole cell B7A provided 50% protection against MSD following B7A challenge; however, no protection was observed in subjects receiving serum antibodies targeting CS6. The lack of observed efficacy in this group may be due to low CS6 surface expression on B7A, the high dose challenge inoculum and/or the use of serum derived antibodies versus colostrum-derived antibodies.

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Introduction

Enterotoxigenic *Escherichia coli* (ETEC), one of several pathotypes of diarrheagenic *E.coli*, causes a secretory diarrhea that can range in presentation from mild discomfort to cholera-like purging. ETEC-mediated diarrhea involves colonization factors (CFs) promoting bacterial adherence to and colonization of the small intestine. This is followed by secretion of one or both of the two enterotoxins (heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT)) that induce fluid and electrolyte secretion resulting in watery diarrhea.[1-4]

Evidence substantiating CFs as protective antigens comes from a number of epidemiological studies as well as controlled human infection models (CHIM).[4-8] This is further substantiated by studies demonstrating passive oral administration of hyperimmune bovine immunoglobulin (BIgG) generated against inactivated whole cell ETEC and against purified CFs protects against moderate to severe diarrhea (MSD) in CHIM using ETEC strains expressing homologous CFs.[9-12]

CS6 is an atypical polymeric antigen that is highly prevalent among ETEC disease isolates globally.[4,13,14] Individuals naturally infected with CS6-expressing ETEC strains exhibit mucosal and serologic immune responses against CS6;[15-17] however, despite data supporting the role of CS6 as a key CF in ETEC-mediated disease, immunological correlates of protection are

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lacking. We sought to assess if CS6 is a protective antigen against CS6-expressing ETEC mediated-diarrhea. Using recombinant CS6, hyperimmune bovine serum IgG (BSIgG) targeting CS6 was derived and assessed for efficacy using previously established models for immunoprophylaxis against ETEC in a CHIM.[11,18]

Methods

Passive vaccination-challenge trial design

Healthy non-pregnant adult subjects aged 18–50 years were recruited from the Mid-Atlantic area. Consented subjects were evaluated to assure good health and eligibility through medical history, physical examination, and screening laboratory tests. Eligible subjects were admitted in two cohorts to the inpatient research unit at the Johns Hopkins University (JHU) Center for Immunization Research Bayview facility. On admission, subjects were block randomized 1:1:1 (20 subjects per group; block size = 6) to receive anti-CS6 BSIgG, anti-B7A whole cell killed BSIgG, or a placebo control (non-hyperimmune BSIgG) in a double-blinded fashion. All investigators, data collectors, and subjects were blinded to treatment allocation.

Study oversight

This clinical trial was approved by the Johns Hopkins Bloomberg School of Public Health IRB in compliance with all federal regulations governing the protection of human volunteers. Clinicaltrials.gov registration NCT03040687.

Study dosing

Two days prior to challenge, subjects began their assigned treatment, thrice daily, 15 minutes after each meal. Each unit dose consisted of approximately 1 gram of total protein of anti-CS6 BSIgG, anti-B7A whole cell BSIgG or the non-hyperimmune BSIgG placebo dose dissolved in 150 mL of sodium bicarbonate buffer (13.35 gm of sodium bicarbonate in 1000 mL of sterile water).

On the day of the challenge, subjects followed the prescribed routine through breakfast and then fasted for 90 minutes. One minute pre-challenge, subjects drank 120 mL sodium bicarbonate buffer, then received approximately 1×10^{10} CFUs of ETEC strain B7A diluted in 30 mL sodium bicarbonate buffer. Subjects received a second dose of BSIgG 15 minutes after challenge, but otherwise fasted for 90 minutes following the challenge. Subjects then received their assigned treatment on the evening of the challenge and then three times daily for an additional four days or until receiving antibiotic treatment.

Subjects were actively monitored for adverse events including signs and symptoms of gastrointestinal illness, rehydrated as needed, and all stools were collected and assessed as previously described.[19-21] Stool (or rectal swab) was plated directly on MacConkey (Mac) agar or Mac agar supplemented with 25ug/ml chloramphenicol (Mac+CM) as strain B7A is resistant to chloramphenicol. Lactose positive colonies were screened for agglutination in anti-B7A whole cell antiserum. Based on the percentage of positive reactions, the CFUs of B7A per gram of stool were estimated. If no B7A colonies were found on the Mac+CM places, additional colonies from the Mac plates were screened.

All subjects initiated a three day course of antibiotics (ciprofloxacin 500 mg orally twice daily) five days after challenge or earlier as indicated per protocol. Subjects were discharged from the unit when clinical symptoms were resolved or resolving and upon culture confirmation of ETEC challenge strain clearance.

Definitions and trial endpoints

The primary endpoint for this study was MSD defined as the following: ≥4 loose/liquid stools or ≥401 grams of loose/liquid stool in any 24 hour period post challenge. Secondary objectives included immune responses to key antigens and a variety of clinical endpoints to include duration and burden of ETEC colonization, total number and volume of stools, maximum 24 hour stool output, and ETEC attributable associated symptoms (to include nausea, vomiting, abdominal pain/cramps). An ETEC disease severity score was calculated using the algorithm described by Porter *et al*[22].

Antigens for bovine immunization

CS6

Recombinant CS6 (Lot 0840) was manufactured under cGMP conditions in Jan 2001 at the Walter Reed Army Institute of Research (WRAIR) Pilot Bioproduction Facility (PBF). The CS6 was derived from ETEC strain E8775 as previously described[23] and as per the Supplemental materials. It is stored in a phosphate buffer, at a concentration of 2.56 mg/ml with an endotoxin content of 60 EU/ml.

Inactivated whole-cell B7A ETEC

Whole cell ETEC strain B7A cells were grown in colonization factor antigen (CFA) broth[24] CS6 expression was confirmed using a CS6-specific inhibition ELISA.[25] The cells were harvested, inactivated, washed and quantified as described in the Supplemental Methods.

Production of bovine serum immunoglobulin products

Bovine immunization

Anti-CS6 BSIgG, anti-whole cell killed B7A BSIgG, and non-hyperimmune BSIgG, were manufactured at SAB Biotherapeutics Inc, Sioux Falls, SD. Cattle were prescreened, randomized and vaccinated five times at four week intervals with either 2 mg of recombinant CS6 (Lot 0840) formulated with SAB proprietary adjuvant SAB-adj-1 or 3×10^{10} CFU killed whole cell B7A formulated with SAB proprietary adjuvant SAB-adj-2. Additional details are included in the supplemental materials. All animal work was conducted under Institutional Animal Care and Use Committee (IACUC) approved protocol 68–000143.

Hyperimmune plasma collection

In dedicated rooms, plasma was collected and separated from four cattle per vaccination group at days 8, 11, and 14 following the fourth and fifth vaccinations under sterile conditions by using an automated plasmapheresis system (Baxter Healthcare, Autopheresis C Model 200). Additional details are included in the supplemental materials. Briefly, plasma collected from the individual animals were measured by ELISA for antibody titers against the B7A ETEC strain, CS6, and O148 lipopolysaccharide (LPS).

Negative control plasma (Non-hyperimmune)

Bovine plasma was collected from four non-immunized cattle using the same method described for the immunized animals. Up to 2.1% of body weight of nonimmune plasma per animal was collected every three to four weeks. The final liquid product was assessed for quality and stored at −20°C ± 5°C. For the negative control, the plasma from the three animals with the lowest background IgG titers against B7A and CS6 were pooled.

Bovine IgG enrichment

The three independent pooled plasma products (anti-B7A, anti-CS6, non-immunized negative control) then underwent a bovine IgG enrichment process in a cGMP clinical manufacturing facility. Each pooled plasma product was thawed at 18-30° C and the pH was adjusted to pH 4.5–4.9 with 20% acetic acid (USP grade). Then the pH adjusted plasma was fractionated with caprylic (octanoic) acid (CA), which selectively precipitates non-Ig bovine plasma proteins (BSA and blood coagulation proteins). After fractionation, the plasma was passed through a depth filtration system to remove non-Ig proteins. The CA-fractionated filtrate was collected into a sterile container. The resulting bulk solution was formulated in USP-grade PBS (1.42 g/L of Na_2HPO_4, 0.24 g/L KH_2PO_4, 7 g/L KCl, pH 7.3–7.5) by diafiltration and addition of PBS and was then concentrated to the desired final protein concentration, 65–80 mg/mL. The final bulk solution was filtered into sterile bioprocess bags (10–20 L) using a 0.22 μm filter. The final Bovine serum IgG bulk product was then filled into 250 mL sterile Nalgene™ multi-dose bottles and stored at −20°C ± 5°C.

Characterization of bovine serum IgG products (BSIgG)

A panel of quality control tests were performed on the final liquid products. The protein content was measured, aggregation assessed, IgG bands in the products were identified, bacterial contamination and endotoxin contents measured, and IgG titers to CS6 and whole-cell B7A quantified. Additional details are found in the Supplemental Materials.

Bacterial challenge strain preparation

The B7A strain, Lot 0481 was manufactured under cGMP at the WRAIR PBF in October 1997. Each vial contains ~9 x 10^8 colony forming units (CFUs) of live B7A (O148:H28- $CS6^+$ LT^+ST^+) in Luria Broth (LB) with 15% glycerol as cryopreservative. Approximately 48 hours prior to the scheduled inoculation, the contents of a single vial were plated for isolation onto CFA agar plates (without bile salts). The plates were incubated for 22–24 hours at 37°C ± 1°C and single colonies were selected and suspended in 3 mL sterile saline (0.9%). The suspension was re-plated on CFA agar plates which were then incubated at 37°C. Cells were harvested in sterile saline after 18–20 hours. Cell density was determined by optical density and the suspension was adjusted to correspond to approximately 1×10^{10} CFUs/ml. Actual CFUs/ml were determined by pre- and post-challenge dilution plating of a sample of the challenge inoculum and averaging the two. The percentage of colonies expressing CS6 was estimated by colony blots developed with rabbit anti-CS6 antisera (provided by NMRC) and goat anti-rabbit IgG conjugated to horse radish peroxidase (Pierce catalog no. 31460). CS6 expression was observed ≥90% of colonies tested in all preparations (data not shown).

Serum antibody responses

Serum was collected three days before challenge and on days 7 and 28 after challenge and stored at −20°C until use. Serum IgA and IgG antibody titers against CS6, LT, and LPS were determined by ELISA as previously described.[26] Seroconversion was defined as a ≥ 4 fold increase over baseline.

Antigen in lymphocyte supernatant (ALS)

Fresh isolated peripheral blood mononuclear cells were resuspended in complete RPMI (10% heat-inactivated fetal calf serum, 1% Penicillin-Streptomycin (Life Technologies) and 1% GlutaMAX (Life Technologies) and incubated in duplicate in 24-well plates (Corning Inc., Corning, NY) at 5×10^6 cells/mL, 1 mL/well, at 37°C and 5% CO_2 for 72 h. Following incubation, culture supernatants were collected and stored at −80°C until tested by ELISA. Abn ALS response was defined as a ≥ 4 fold increase over baseline.

Statistical analysis

The prevalence of adverse events was compared between each active group and the placebo group using Fisher's exact test. Continuous (and ordinal) variables were similarly compared using the Kruskal-Wallis test. Protective efficacy was calculated as (1 − relative risk). The null hypotheses were that there would be no difference in the post-challenge moderate-severe diarrhea rates between subjects receiving non-hyperimmune BSIgG and either group receiving hyperimmune BSIgG. Based on a Fisher's exact test with a 1-sided alpha = 0.05, a sample size of 20 subjects per group yielded an 88% power to detect a 60% reduction in moderate-severe diarrhea rates presuming at least an 80% moderate-severe diarrhea rate in subjects receiving non-hyperimmune BSIgG. All other comparisons were made using a 2-sided alpha = 0.05. There were no adjustments for multiple comparisons.

Results

One hundred and eighteen subjects were recruited and screened for participation, of whom 68 were eligible. A total of 60 subjects were admitted to the inpatient research ward, randomized, and started on anti-CS6 (n = 20), anti-B7A whole cell killed (n = 20) or non-hyperimmune (n = 20) BSIgG (Supplemental Figure 1). No baseline differences were noted across groups in terms of age, sex, or ethnicity (Table 1). Prior to challenge, one subject from the anti-CS6 group was discharged from the inpatient facility due to health events unrelated to the investigational products. All other subjects were compliant with oral immunoprophylaxis and all doses were taken within fifteen minutes after each meal. Adverse events (AEs) determined to be at least possibly related to the BsIgG were infrequent and mild with the exception of one case of moderate severity abdominal cramping. Reported adverse events were predominantly

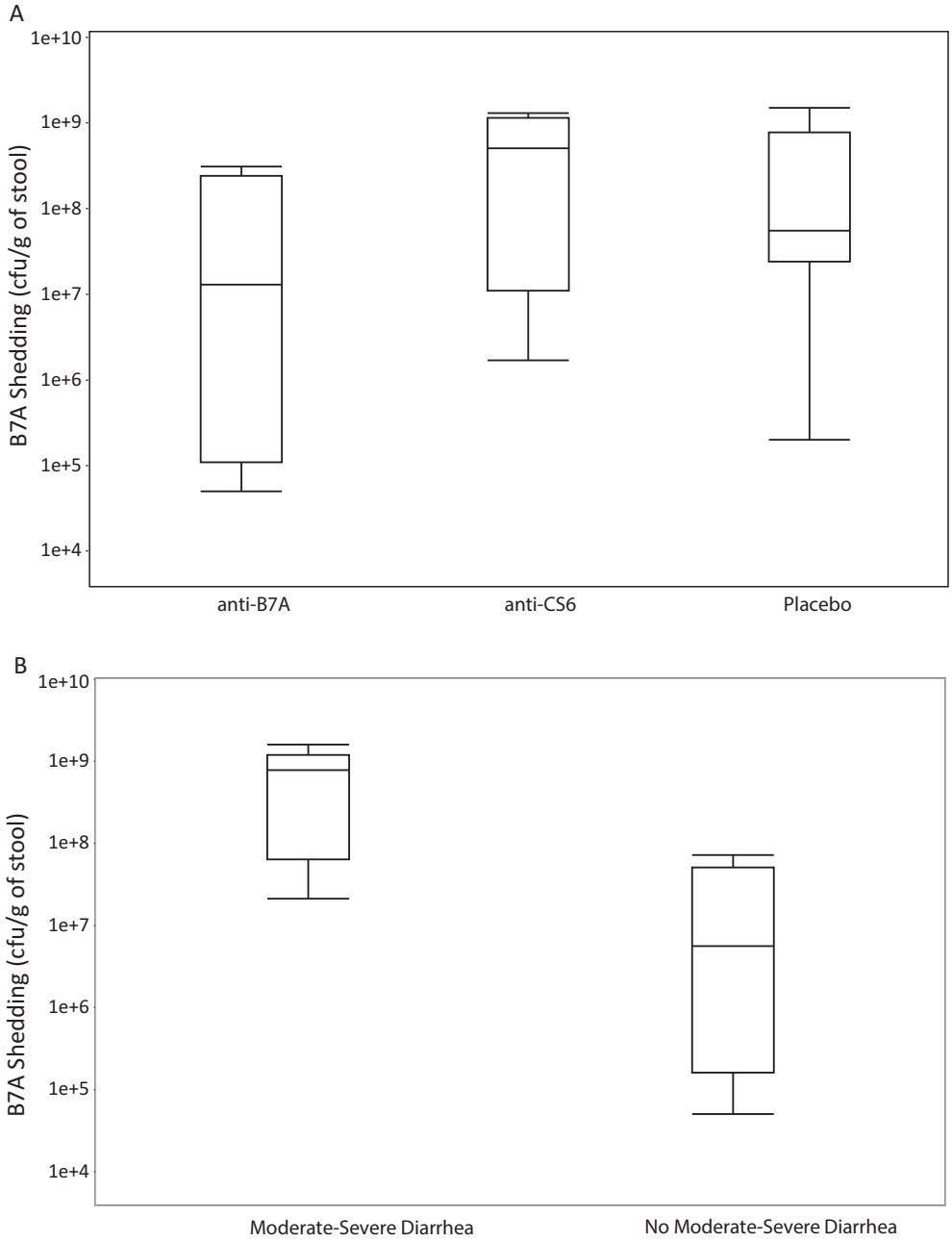

Figure 1. Quantitative shedding of ETEC strain B7A on two days after oral challenge.

Legend: Distribution of data presented as a box and whisker plot in which the horizontal line represents the median value, the ends of the boxes represent 1st and 3rd quartiles and the whiskers represent the 1st and 3rd quartiles ± 1.5*(interquartile range) Panel a. Number of colony forming units of ETEC strain B7A isolated per gram of stool from subjects by treatment group. Panel b. Number of colony forming units of ETEC strain B7A isolated per gram of stool from subjects stratified by whether or not the subject met the primary endpoint of moderate-severe diarrhea.

gastrointestinal in nature with flatulence (15.3%) the most common across groups (Table 2).

After challenge with 1.5 to 1.7 × 10^{10} CFUs of strain B7A, 70.0% (14/20) of subjects receiving non-hyperimmune BSIgG met the primary endpoint of moderate-severe diarrhea (Table 3). In contrast, 35.0% (7/20) of subjects receiving anti-B7A BSIgG had moderate to severe diarrhea yielding a 50% (95% confidence intervals (CI): 3.0%, 74.2%) protective efficacy (Fisher's exact 1-sided p = .03). A total of 12/19 (63.2%) subjects receiving anti-CS6 BSIgG had moderate to severe diarrhea yielding a 9.8% (95% CI: −41.2%, 42.3%) protective efficacy (Fisher's exact 1-sided p = .45).

Table 1. Demographic characteristics of subjects completing the passive prophylaxis series and receiving the ETEC B7A challenge.

	Non-Hyperimmune	Anti-CS6	Anti-B7A
N	20	19	20
Age, Standard Deviation	34.8 (8.8)	32.2 (8.5)	35.9 (8.0)
Gender, N (%)			
Female	9 (45.0)	8 (41.2)	4 (20.0)
Male	11 (55.0)	11 (57.9)	16 (80.0)
Race, N (%)			
African-American	15 (75.0)	14 (73.7)	18 (90.0)
Caucasian	3 (15.0)	5 (26.3)	2 (10.0)
Other	2 (10.0)	0 (0.0)	0 (0.0)
Ethnicity, N (%)			
Hispanic or Latino	3 (15.0)	3 (15.8)	1 (5.0)
Not Hispanic or Latino	17 (85.0)	16 (84.2)	19 (95.0)

Table 2. Adverse events determined by the clinical investigator to be at least possibly related to the BsIgG products.

	Group			
AE Description	Placebo (n = 20)	Anti-CS6 (n = 19)	Anti-B7A (n = 20)	Total (n = 59)
Abdominal cramps, n(%)	1 (5.0)	2 (10.5)	0 (0.0)	3 (5.1)
Anorexia, n(%)	0 (0.0)	0 (0.0)	1 (5.0)	1 (1.7)
Bloating, n(%)	0 (0.0)	1 (5.3)	2 (10.0)	3 (5.1)
Flatulence, n(%)	2 (10.0)	3 (15.8)	4 (20.0)	9 (15.3)
Headache, n(%)	0 (0.0)	0 (0.0)	2 (10.0)	2 (3.4)
Nausea, n(%)	0 (0.0)	0 (0.0)	1 (5.0)	1 (1.7)
Urgency, n(%)	1 (5.0)	0 (0.0)	0 (0.0)	1 (1.7)

All reported symptoms were mild except for 1 moderate episode of abdominal cramps.

Table 3. Signs and symptoms of ETEC following challenge with 1.7 to 1.5 × 10^{10} colony forming units of strain B7A.

	Group		
	Placebo (n = 20)	Anti-CS6 (n = 19)	Anti-B7A (n = 20)
Moderate-Severe Diarrhea (%)	70.0	63.2	35.0[a]
Abdominal distension (%)	45.0	10.5[b]	10.0[b]
Abdominal pain (%)	60.0	63.2	35.0
Abdominal tenderness (%)	35.0	26.3	0.0[b]
Arthralgia (%)	25.0	10.5	0.0[b]
Chills (%)	25.0	36.8	0.0[b]
Decreased appetite (%)	50.0	52.6	20.0
Headache (%)	50.0	36.8	20.0
Malaise (%)	45.0	42.1	5.0[b]
Myalgia (%)	30.0	31.6	15.0
Nausea (%)	55.0	63.2	0.0[b]
Fever (%)	0.0	10.5	5.0
Vomiting (%)	25.0	15.8	5.0
Median (Q1, Q3) maximum 24 hr loose stool weight (g)	483.5 (110.0, 929.5)	451.0 (92.0, 879.0)	349.5 (0.0, 634.0)
Median (Q1, Q3) maximum 24 hr loose stool frequency	4.0 (1.0, 7.5)	4.0 (1.0, 8.0)	2.0 (0.0, 5.0)
Median (Q1, Q3) total loose stool output weight (g)	745.5 (309.0, 1371.0)	764.5 (340.0, 1160.0)	659.0 (297.0, 1154.0)
Median (Q1, Q3) total frequency of loose stools	7.5 (2.0, 10.0)	5.5 (2.0, 13.0)	4.0 (3.0, 12.0)
Median (Q1, Q3) ETEC disease severity	4.0 (2.0, 5.5)	4.0, (3.0, 5.0)	3.0 (1.0, 3.5)[c]

[a] $p < 0.05$ compared to placebo (based on a 1-sided Fisher's Exact test).
b $p < 0.05$ compared to placebo (based on a 2-sided Fisher's Exact test).
c $p < 0.05$ compared to placebo (based on a 2-sided Kruskal-Wallis test).

Additionally, subjects receiving anti-B7A BSIgG had lower rates of several other signs/symptoms of ETEC infection as well as a lower ETEC disease severity score compared to non-hyperimmune BSIgG recipients (Table 3). There was also a non-significant reduction in the frequency and volume of loose stool output in subjects receiving anti-B7A BSIgG compared to the other study groups.

All subjects shed the B7A in their stool with no significant differences in quantity of shedding at Day 2 when comparing treatment groups to placebo (anti-CS6 p = .55; anti-B7A p = .13); however, there appeared to be a trend toward reduced shedding in subjects receiving anti-B7A BSIgG compared to non-hyperimmune BSIgG recipients (Figure 1a). Among all subjects, those who had moderate-severe diarrhea shed significantly ($p < .0001$) more B7A on Day 2 than subjects with mild or no diarrhea (Figure 1b).

Anti-CS6 serologic and ALS response rates were low (≤30%) across all study groups (Table 4). Interestingly, prophylaxis with anti-CS6 (p = .05) and anti-B7A (p = .06) BSIgG appeared to blunt anti-CS6 serum IgG responses by day 7 compared to subjects receiving non-hyperimmune serum (Figure 2a). Anti-CS6 serum IgA was less affected by the oral prophylaxis (Figure 2b) but appeared somewhat blunted as well. Post-challenge anti-LT responses as assessed by serology and ALS ranged from 21.1% to 63.2% in subjects receiving anti-CS6 BSIgG, 5.0% to 35.0% in subjects receiving anti-B7A BSIgG, and 25.0% to 65.0% in subjects receiving the placebo BSIgG (Figure 2c, Table 4). Additionally, as shown in Figure 2d, by Day 28, lower anti-LT IgA titers in were observed subjects treated with anti-CS6 or anti-B7A BSIgG compared to the subjects receiving non-hyperimmune serum (p = .02 and p = .01, respectively). There was no apparent difference in anti-LT IgG titers. The most frequent immune response was to LPS. Specifically, ALS IgA response to LPS was noted in 100.0% of anti-CS6 BSIgG recipients, 80.0% of

Table 4. Number and proportion of subjects developing a ≥ 4-fold rise in serologic or ALS responses following controlled human infection with ETEC strain B7A.

		Study Group			
Antigen	Assay	anti-CS6 (N = 19) n (%)	anti-B7A (N = 20) n (%)	Placebo (N = 20) n (%)	All (N = 59) n (%)
Coli Surface Antigen 6 (CS6)	Serology (IgA)	1 (5.3%)	0 (0.0%)	3 (15.0%)	4 (6.8%)
	Serology (IgG)	1 (5.3%)	3 (15.0%)	6 (30.0%)	10 (16.9%)
	ALS (IgA)	2 (10.5%)	2 (10.0%)	6 (30.0%)	10 (16.9%)
	ALS (IgG)	1 (5.3%)	3 (15.0%)	6 (30.0%)	10 (16.9%)
Heat-Labile Toxin (LT)	Serology (IgA)	6 (31.6%)	1 (5.0%)	10 (50.0%)	17 (28.8%)
	Serology (IgG)	12 (63.2%)	7 (35.0%)	12 (60.0%)	31 (52.5%)
	ALS (IgA)	4 (21.1%)	2 (10.0%)	5 (25.0%)	11 (18.6%)
	ALS (IgG)	7 (36.8%)	4 (20.0%)	7 (35.0%)	18 (30.5%)
Lipopolysaccharide (LPS) Type 0148	Serology (IgA)	14 (73.7%)	11 (55.0%)	15 (75.0%)	40 (67.8%)
	Serology (IgG)	7 (36.8%)	8 (40.0%)	10 (50.0%)	25 (42.4%)
	ALS (IgA)	19 (100.0%)	16 (80.0%)	18 (90.0%)	53 (89.8%)
	ALS (IgG)	13 (68.4%)	11 (55.0%)	14 (70.0%)	38 (64.4%)

anti-B7A BSIgG recipients, and 90.0% of placebo recipients. The levels of anti-LPS IgG and IgA antibodies were unaffected by either treatment compared to the levels observed in the placebo group (Figure 2e,f).

Discussion

Travelers' diarrhea remains a significant threat with ETEC a primary bacterial pathogen cause.[27,28] Vaccines or therapeutics against ETEC will need to target different strains expressing various CFs to ensure a sufficient breadth of coverage. This study was designed to evaluate whether orally administered bovine serum antibodies targeting various ETEC antigens (a CF and the whole cell) would be able to prevent or reduce moderate to severe diarrhea following challenge with the CS6-expressing ETEC strain B7A. The products were safe and well-tolerated, similar to other studies with orally administered bovine antibodies.[9-12,18] Additionally, antibodies targeting the B7A whole cell yielded a 50% reduction in MSD and a reduced disease severity score; however, anti-CS6 yielded no significant protection against MSD.

There are several potential reasons for the lack of observed efficacy. First, it is possible that despite epidemiologic associations between CS6-expressing strains and human disease,[29-31] CS6 does not play a critical role in disease in the ETEC CHIM. However, preclinical studies, including a non-human primate model, demonstrate vaccination with CS6-derived antigens co-administered with an LT-based adjuvant confers protection against diarrhea following challenge with B7A.[32] While beyond the scope of this work, it would be of interest to compare moderate to severe diarrhea in the CHIM between a wildtype B7A strain and an isogenic mutant that fails to express CS6 to better understand the role of CS6 in a controlled infection.

If CS6 is an important virulence factor, there are other possibilities for the lack of observed protection in subjects receiving anti-CS6 antibody. The CS6 clone used to generate the antigen delivered to the cattle was from ETEC strain E8775[33] whereas the challenge strain in the CHIM was strain B7A. The CssA and CssB subunits between these two strains share 92% and 96% identity, respectively, at the protein level (GenBank accession numbers as follows: E8775 CssA: AAB51361.1; E8775 CssB: AAB51362.1; B7A CssA: EDV60821.1; B7A CssB: EDV60824.1). Sabui et al reported that specific alleles of the CS6 subunits were strongly associated with children presenting with diarrhea or non-diarrheagenic controls, raising the possibility that CS6 variation may affect host binding and subsequent disease.[34] It is possible that a critical, protective residue involved in B7A pathogenesis may not be expressed by E8775, and thus the anti-CS6 antibody product may have not had the capacity to prevent B7A disease.

It is also possible that serum derived antibodies are incapable of providing robust protection compared to the colostral antibodies we have utilized previously.[11,18] To our knowledge, this is the first attempt to utilize orally delivered bovine serum antibodies to protect against an enteric infection. It may be that colostrum-derived antibodies, due

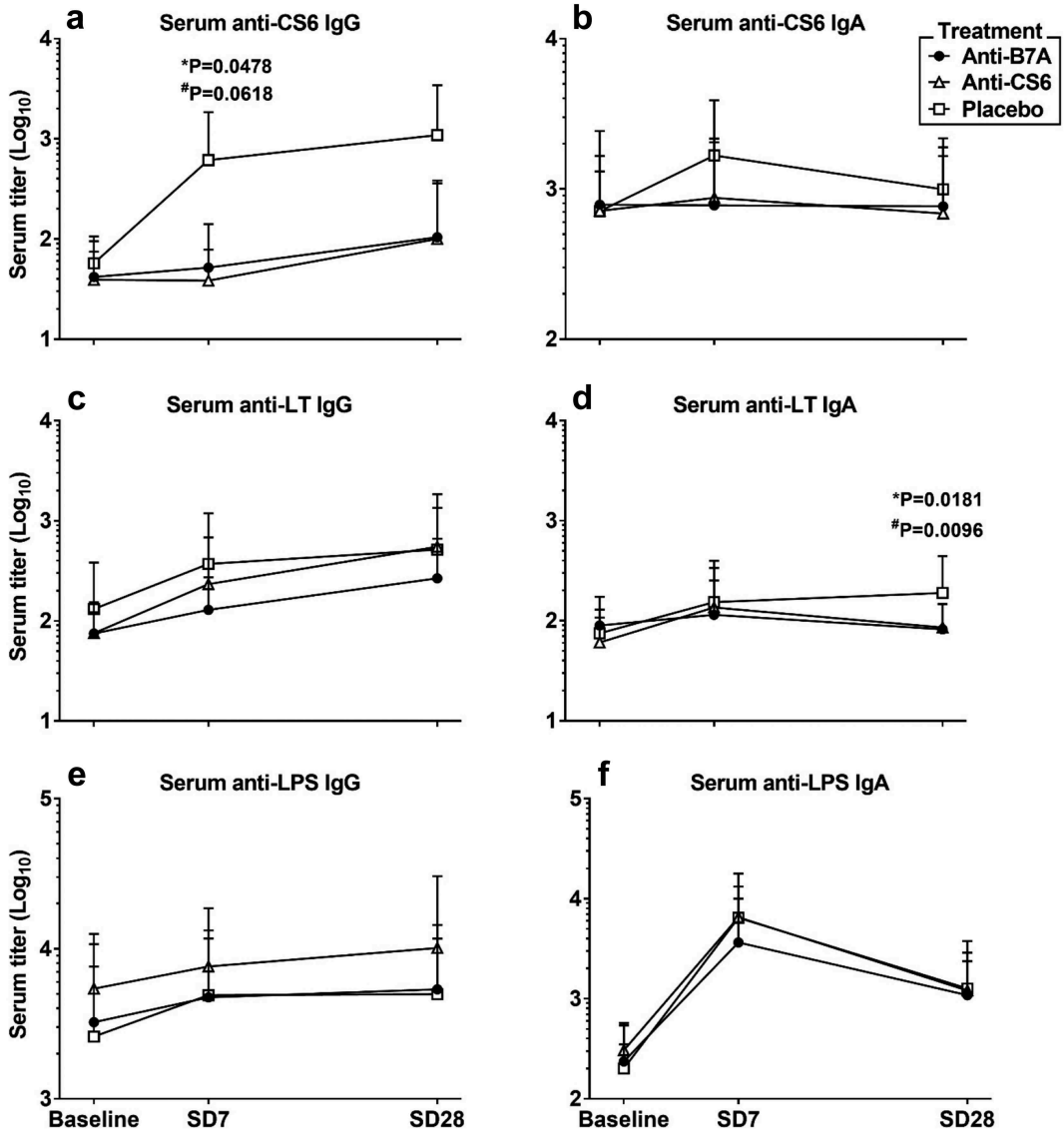

Figure 2. Serologic responses to oral challenge with ETEC strain B7A stratified by treatment group.

Footnote Volunteers were orally treated between days −3 before challenge to day +2 with BSIgG contained anti-B7A killed whole cell, or anti-CS6, or placebo antibodies. Serum samples were collected prior to challenge (baseline) and on days 7 and 28 after the challenge. Statistical comparisons were performed by Mann-Whitney test and considered significantly different when $p < .05$; *for comparisons between Anti-CS6 and Placebo groups at the same time point; #for comparisons between Anti-B7A and Placebo groups at the same time point.

to their intrinsic nature, are able to survive the orogastric delivery route better, thereby providing more active antibody to the gut.[35] While not addressed in this study, it would be of value to determine the amount of functional antibody available in the gut following ingestion.

Consistent with the data seen here, we previously observed a low number of anti-CS6 responders after oral challenge with CS6+ ETEC B7A strain (K. Talaat et al, submitted for publication). Interestingly, the proportion of responders and the magnitude of responses were higher among the volunteers in the placebo group, compared to volunteers treated with anti-CS6 or B7A BSIgG, possibly suggesting that both products partially blunted the anti-CS6 response. It seems plausible to speculate that, contrary to CS6 or LT, blunting of the anti-LPS response by oral prophylaxis is rather difficult, given LPS represents a major component of the bacterial cell.

These data build on prior findings that passive immunoprophylaxis targeting ETEC, including

products directed against the colonization factors, can reduce ETEC-mediated disease.[9-12] Interestingly, in this study, we did not see significant protection with a product designed against the major colonization factor of B7A, CS6, whereas the anti-whole cell product did provide protection. The lack of protection provided by the anti-CS6 product may be specific to the B7A challenge strain, or it may reflect a different role or function of this CF as compared to some of the others (e.g. CFA/I) on the surface of the bacteria that complicates passive protection in this setting. Alternatively, there may be a role for other antigens, such as CS21 which is also expressed on B7A, in combination with CS6 which may be important and points to the potential value in immune proteomics in identifying a protective immunologic profile.[36] This may need to be explored further as multivalent vaccines and therapeutics are developed for ETEC.

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No potential conflicts of interest were disclosed

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