BRIEF REPORT

Volunteer Challenge With Enterotoxigenic *Escherichia coli* That Express Intestinal Colonization Factor Fimbriae CS17 and CS19

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Human challenges with enterotoxigenic *Escherichia coli* (ETEC) have broadened our understanding of this important enteropathogen. We report findings from the first challenge studies using ETEC-expressing colonization factor fimbria CS17 and CS19. LSN03-016011/A (LT, CS17) elicited a dose-dependent effect, with the upper dose (6×10^9 organisms) causing diarrhea in 88% of recipients. WS0115A (LTSTp, CS19) also showed a dose response, with a 44% diarrhea rate at 9×10^9 organisms. Both strains elicited homologous antifimbrial and anti-LT antibody seroconversion. These studies establish the relative pathogenicity of ETEC expressing newer class 5 fimbriae and suggest suitability of the LT|CS17-ETEC challenge model for interventional trials.

Received 27 August 2010; accepted 23 February 2011.

Potential conflicts of interest: none reported.

Presented in part: The 4th International Conference on Vaccines for Enteric Diseases, 25–27 April 2007, Lisbon, Portugal, Poster 13.

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The Journal of Infectious Diseases 2011;204:60–4

Published by Oxford University Press on behalf of the Infectious Diseases Society of America 2011.

0022-1899 (print)/1537-6613 (online)/2011/2041-0010$14.00

DOI: 10.1093/infdis/jir220

For certain human infectious diseases, volunteer challenge models have been developed as tools to study disease pathogenesis and assess new treatment and prevention measures. Enterotoxigenic *Escherichia coli* (ETEC), a predominant global cause of bacterial diarrhea, have been extensively studied in this manner, but with a limited number of strains. Strains expressing both the heat-labile (LT) and heat-stable (ST) enterotoxins, LT-only, and ST-only have induced diarrhea in volunteers, attesting to the clinical significance of each of these enterotoxins [1]. In addition, ETEC strains expressing the earliest recognized colonization factors, namely colonization factor antigen I (CFA/I) fimbriae, coli surface antigen (CS) 6, and coexpressed CS1 and CS3, are pathogenic in volunteers [2, 3]. Subsequent ETEC volunteer challenge studies assessing new antimicrobial treatment and prophylaxis regimens and investigational vaccine efficacy have primarily involved the use of a small number of highly characterized strains. The most recent ETEC challenge model strain, E24377A (LTST, CS1+CS3), was established in 1984 [4].

Many ETEC colonization factors (CFs) have since been discovered, including several that are genetically related to CFA/I, archetype of class 5 fimbriae. Although epidemiological data have incriminated some of these CFs as virulence determinants, the lack of a standardized, clinically relevant animal model of human-derived ETEC diarrhea has precluded a more definitive causal linkage with disease. Two newer ETEC class 5 fimbrial types of interest are CS17 and CS19 fimbriae. Discovered in 1990, CS17 is strongly associated with the LT-only toxin phenotype, shows a wide geographic distribution [5, 6], and has been independently linked with illness [7]. First described in 1997, CS19 fimbriae are closely related to CS17 [8], associated with LT and LTST phenotypes, and have been isolated in multiple studies in Asia and Africa [9].

In the work presented here, we sought to establish the pathogenicity of CS17-ETEC and CS19-ETEC strains in the volunteer challenge model. In developing these new models, a major aim has been to expand the available number of well-defined ETEC with distinct toxin-CF phenotypes that can be used in the early evaluation of new generation investigational ETEC vaccines.

METHODS

CS17- and CS19-Expressing ETEC

The 3 strains selected for experimental challenge were isolated from stool samples from individuals with diarrhea (Table 1). These strains were expanded into master cell banks under

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
FEB 2011		**00-00-2011 to 00-00-2011**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Volunteer Challenge With Enterotoxigenic Escherichia coli That Express Intestinal Colonization Factor Fimbriae CS17 and CS19	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Naval Medical Research Center,Enteric Diseases Department,503 Robert Grant Avenue,Silver Spring,MD,20910**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**Same as Report (SAR)**	**5**	

Table 1. Toxin Genotypes and In Vitro Enterotoxin Production Levels for ETEC Strains Used in Volunteer Challenge Experiments

	Strain information					LT		ST	
Strain	Year of isolation	Country of origin	Case profile	Serotype[a]	CF type[b]	Genotype[c]	Production (ng/mL)[d, e]	Genotype[c]	Production (ng/mL)[d, e]
LSN03-016011/A	2003	Turkey	U.S. expatriate	O8:H-	CS17	+	59	–	nd[f]
WS0115A	1993	Egypt	Infant	O114:H-	CS19	+	60	+ (STp)	129
DS26-1	1990	Saudi Arabia	U.S. soldier	O8:H9	CS19	+	8	–	Nd

NB, All strains were susceptible to ciprofloxacin and trimethoprim-sulfamethoxazole.

[a] Serotype was determined by classic serological methods at the Universidad Nacional Autónoma de México (UNAM). H- indicates non-motility.

[b] CF identity was initially determined by colony immunoblot analysis with CF-specific monoclonal antibodies, and confirmed by DNA sequence analysis of the fimbrial major subunit and Western blot analysis of bacterial heat extracts with monospecific rabbit antisera against each CF.

[c] Toxin genotypes were confirmed by Southern blot hybridization with plasmid preparations from each strain, using probes specific for LT STp, and STh [10].

[d] Quantitation of ST and LT was performed using GM1-ELISA methods [11]. Bacteria were grown in LB liquid culture at 37°C overnight for the assay. The reported concentrations of ST and LT were measured in culture supernatants and sonicates, respectively. Toxin values were interpolated from a standard curve using known quantities of each purified toxin. Strain DS26-1 produced 20 ng/ml of LT in the culture supernatant, the only one for which this value was higher than that in the corresponding sonicate preparation.

[e] ETEC strain H10407 (LTSThSTp; CFA/I; O78:H11), a well-characterized and highly pathogenic strain, was used as a reference control in the enterotoxin assays, producing 66 and 99 ng/ml of LT and STa, respectively.

[f] Not determined.

current Good Manufacturing Practices (GMP) at the Walter Reed Army Institute of Research Pilot Bioproduction Facility.

Experimental Infection

The study protocol was approved by the Western and Naval Medical Research Center institutional review boards and was conducted in compliance with all applicable federal regulations governing the protection of human participants. Signed, informed consent was obtained on all participants before entry.

This open-label, strain, and dose-finding study was designed to establish a human challenge model for CS17- and CS19-ETEC that caused a ≥80% diarrhea attack rate. Strains were fed to successive cohorts of participants beginning with a dose of 5×10^8 colony-forming units (cfus). The maximum administered dose was 9×10^9 cfu.

Approximately 48 h before challenge, a GMP master cell bank vial was thawed, streaked onto CFA (with bile salts) [12] and MacConkey agar plates, and incubated for 22–24 h at 35–37°C. Ten CFA agar colonies that agglutinated with homologous CF antiserum were suspended in sterile saline (0.85% sodium chloride). This suspension was used to inoculate CFA agar plates supplemented with bile salts. After incubation at 35–37°C for 18–20 h, bacteria were harvested in sterile saline, and the suspension was diluted in phosphate-buffered saline to the appropriate concentration according to optical density at 600 nm. The suspension was examined for purity by Gram stain and for CF expression by agglutination with homologous CF antiserum. Bacterial concentration was quantified by the serial dilution and plating method using Luria agar plates.

Healthy male and nonpregnant female participants (age, 18–45 years) were admitted to the Johns Hopkins University General Clinical Research Center. History of travel to a developing country in the preceding 2 years was exclusionary. The morning after admission, participants ate a light breakfast, fasted for 90 min, and then ingested 120 mL of 1.33% sodium bicarbonate buffer to neutralize stomach acidity. One minute later, they ingested a dose of ETEC in 30 mL of the same sodium bicarbonate solution and fasted for another 90 min.

All stool specimens were collected, examined, and weighed. The first 2 stool specimens each day were cultured. If no stool was passed, a rectal swab was obtained. A swab of stool was inoculated directly onto MacConkey agar and incubated overnight at 37°C. Up to 10 lactose-positive colonies were spotted onto CFA agar [12] and tested by colony-lift immunoblot analysis with challenge strain-specific antiserum.

The primary clinical outcome of diarrhea was defined as ≥2 loose stools in a 48-h period that totaled ≥200 g or a single loose stool of ≥300 g. Diarrhea was graded as mild (1–3 loose stools totaling ≤400 g), moderate (4–5 loose stools or 401–800 g), or severe (≥6 loose stools or >800 g). Signs and symptoms were graded as mild (no interference with activities), moderate (prevented some activities), or severe (prevented most activities).

All participants initiated ciprofloxacin treatment (500 mg twice daily for 3 days) 120 h after challenge unless one of the criterion for earlier treatment was met, including (1) severe diarrhea, (2) moderate diarrhea for 2 consecutive days, (3) mild-moderate diarrhea plus ≥2 of the following symptoms: fever (≥100.5°F), vomiting, abdominal pain or cramps, nausea, headache, and myalgia or arthralgia coded as severe.

Immunology

Serum immune responses were assessed at baseline and 10 and 28 days after challenge. Anti-LT antibody titers were measured using the GM_1–enzyme-linked immunosorbent assay (ELISA) method [13]. Anti-CS17 and anti-CS19 antibody titers were determined using methods described elsewhere [14]. Pre- and

post-dosing serum samples were tested simultaneously and expressed as the geometric mean of duplicate determinations. Seroconversion was defined as a ≥4-fold increase over the prechallenge titer. Reciprocal end point titers <5 were assigned a value of 2.5 for computational purposes.

RESULTS

A total of 38 participants were administered one of the ETEC strains. Participants were mostly male (68%) and African-American (95%), with a median age of 35 years. There were no statistically significant differences in demographic characteristics between the study groups.

An initial cohort of 5 participants received 7×10^8 cfu of the LT|CS17-ETEC strain LSN03-016011/A. Three participants (60%) developed diarrhea: 1 mild and 2 severe (Table 2). A subsequent cohort of 8 participants was challenged at a higher dose (6×10^9 cfu). Seven participants (88%) developed diarrhea (4 moderate and 3 severe). One participant received intravenous fluids for severe diarrhea with nausea and vomiting. The only participant who did not develop diarrhea had severe nausea and moderate abdominal cramps with a single loose stool. Of the 13 participants given LSN03-016011/A, 77% had abdominal pain or cramps and 31% had nausea (Table 2). In total, the targeted threshold attack rate of ≥80% was achieved with LSN03-016011/A at the higher dose.

A cohort of 5 participants was administered 4×10^8 cfu of the LTSTp|CS19-ETEC strain WS0115A. One recipient developed diarrhea (Table 2). A second cohort ($n = 6$) received a higher dose of 3×10^9 cfu, and 2 participants developed diarrhea (both moderate). The relatively low attack rate prompted a switch to DS26-1, which was administered to 5 participants at 4×10^8 cfu. Although 2 participants had loose stools, neither met the diarrhea definition, and model establishment with DS26-1 was suspended. WS0115A was then given to 9 participants at an inoculum of 9×10^9 cfu. Four participants (44%) developed diarrhea, and 3 had loose stools but did not meet the diarrhea outcome definition. All participants shed the organism from the day after challenge until initiation of antibiotics.

All 3 ETEC strains induced anti-CF seroconversion (Table 2), with the highest titers observed in those receiving the CS17-ETEC strain (data not shown). Overall, anti-LT IgG and IgA seroconversion developed in 92% of participants receiving LSN03-016011/A (Table 2). In contrast, anti-LT seroconversion rates for those receiving CS19-ETEC were 44% for IgG and 52% for IgA isotype.

DISCUSSION

Experimental challenge of volunteers with ETEC remains a valuable investigative tool, because of the paucity of applicable animal models and the global health importance of this

Table 2. Clinical Illness and Serologic Response in Subjects Challenged With CS17- and CS19-ETEC Strains

Strain	CF type	Dose (cfu)	*N*	Subjects with diarrhea, %	Time to meeting definition of diarrhea in h[e]	Diarrhea output (g)[a]		Other gastrointestinal signs/symptoms, %			Anti-CF antibody seroconversion, %[b]		Anti-LT antibody seroconversion, %	
						Total	Max. 24 h	Abdominal cramps	Vomiting	Nausea	IgG	IgA	IgG	IgA
LSN03-016011/A	CS17	7×10^8	5	60	14 (7, 25)	787 (384, 1139)	556 (326, 845)	80	40	40	60	100	80	80
		6×10^9	8	88	14 (12, 15)	1001 (517, 1429)	508 (429, 717)	75	37	25	75	75	100	100
WS0115A	CS19	4×10^8	5	20	17	1186	1186	40	0	20	40	60	60	60
		3×10^9	6	33	24 (23, 25)	677 (402, 951)	449 (402, 495)	50	0	33	33	83	50	50
		9×10^9	9	44	64 (26, 102)	692 (461, 919)	462 (407, 549)	22	0	11	44	100	44	55
DS26-1	CS19	4×10^8	5	0	n/a	0	0	0	0	0	0	40	20	40

[a] Median (interquartile range) calculated for subjects who met the study definition of diarrhea. "Total" is the total output of loose stools during the study period, and "max. 24 h" is the maximum output of loose stools in any 24-hour period.

[b] % of subjects with ≥ 4-fold increase in anti-CS17 antibody among those challenged with LSN03-016011/A or anti-CS19 antibody among those challenged with WS0115A or DS26-1.

disease. Since the feasibility of such models was first established in 1971, numerous observational and interventional studies have been performed, mostly with 3 particular strains, H10407 (CFA/I), B7A (CS6) and E24377A (CS1, CS3), all of which produce LT and STh and well-known human CFs. Our aim to establish the pathogenicity of ETEC-expressing CS17 and CS19 fimbriae, 2 more recently recognized and closely related class 5 fimbriae, was achieved. Two corresponding ETEC strains were fed to successive cohorts at ascending doses, and both elicited a dose-dependent incidence of diarrhea. We also sought to establish the clinical basis for the use of these strains in future interventional studies. Attainment of a high attack rate (88%) and lack of any unexpected outcomes after feeding 6×10^9 cfu of the CS17-ETEC strain LSN03-016011/A suggest the suitability of this strain for such purposes. Indeed, establishment of a model with this LT-only ETEC strain adds a useful option for ETEC volunteer challenge studies. In contrast, a 44% diarrhea attack rate at a dose just $<10^{10}$ cfu of WS0115A (CS19) and absence of diarrhea after low-dose challenge with the other CS19-ETEC strain DS26-1 suggest that these 2 strains are less pathogenic than is LSN03-016011/A. These findings weigh against future use of either of the CS19-ETEC strains for interventional studies.

Clinical features distinguished the disease observed in this study from those in prior experimental challenge studies with ETEC strains of similar toxin phenotypes. Similar to LT-only ETEC strains TD255 C4 and E2528C1 [1, 15], LSN03-016011/A elicited nonvoluminous diarrhea with a short incubation period and was frequently accompanied by other gastrointestinal signs and symptoms. On the other hand, the diarrhea attack rate for LSN03-016011/A was double that of these other 2 strains at comparable doses (ie, 10^9–10^{10} cfu) and manifested as a more severe illness. CS19-ETEC strain WS0115A shares the LTST enterotoxin phenotype with several other ETEC that have been repeatedly given to volunteers. Among these, ETEC strains H10407, E24377A, and B7A have generally resulted in very high attack rates and voluminous diarrhea at comparable doses [2, 3]. Of interest, WS0115A is distinguished from these other ETEC prototypes in that it exhibits the STp rather than the STh genotype. Although this association may be circumstantial, population-based studies have suggested that STh-ETEC exhibit greater pathogenicity for humans than do STp-ETEC [9]. The in vitro levels of LT and ST expression by WS0115A were comparable to those of H10407 (Table 1), at least suggesting that lowered toxin expression is an unlikely explanation for the lower pathogenicity observed with WS0115A.

Epidemiologically, LT-only ETEC have shown inconsistent association with disease [9, 16]. Our volunteer findings with LSN03-016011/A, however, add to the weight of evidence that subpopulations of LT-only ETEC are pathogenic. The near invariant association between CS17 and LT-only genotypes and their widespread distribution suggests that this ETEC subpopulation is a human pathogen.

Our findings add to an understanding of the importance of CS17 and CS19 fimbriae as virulence determinants. The great majority of volunteers exhibited 4-fold seroconversion to the homologous CF, indicating in vivo immune recognition. CS17 and CS19 are highly related to one another and fall into the larger genetically defined group of class 5 fimbriae. Strains expressing 5 of the 8 members of this class have now been shown to cause disease in volunteers, supporting the virulence of this class of adhesins and highlighting them as a major consideration for vaccine development.

As the best characterized LT-only ETEC challenge strain, LSN03-016011/A could facilitate vaccine research. For example, a basic benchmark for ETEC vaccines that solely generate anti-LT immunity would be demonstration of protection against disease caused by LT-only ETEC. Of interest, vaccination with one such investigational product, a purified, native LT skin patch, did not confer protection against disease in volunteers challenged with an LTST ETEC strain [3]. Failure may have been attributable to enterotoxin mismatch, insufficiency of induced anti-LT immunity, or an overwhelming challenge dose. A comparable vaccination-challenge study using LSN03-016011/A could be informative in this regard. To be clear, we developed this model for use in the evaluation of bovine hyperimmune antifimbrial and anti-adhesin immunoglobulin preparations specific for CS17. The corresponding findings will be reported elsewhere and substantiate the usefulness of this new model.

Funding

This work was supported by the Peer-Reviewed Medical Research Program (W81XWH-04-1-0067 to SJS) and by Johns Hopkins University School of Medicine General Clinical Research Center (M01-RR00052 from the National Center for Research Resources, National Institutes of Health).

Acknowledgments

We thank David Rockabrand, Sami Farid, and Carl Mason, for their invaluable assistance in characterizing strains and providing early passage stocks of each; Armando Navarro and Alejandro Cravioto, for kindly performing O:H serotype analysis of the challenge strains; Ann-Mari Svennerholm, for contributions to this work in quantitation of in vitro enterotoxin production by the ETEC challenge strains; and the staff of the Johns Hopkins CIR and GCRC, who provided superb clinical support. The opinions and assertions herein should not be construed as official or representing the views of the Department of the Navy, Department of the Army, the Department of Defense, or the U.S. Government. This is a U.S. Government work. There are no restrictions on its use.

References

1. Clements ML, Levine MM, Black RE, et al. Lactobacillus prophylaxis for diarrhea due to enterotoxigenic *Escherichia coli*. Antimicrob Agents Chemother **1981**; 20:104–8.
2. Coster TS, Wolf MK, Hall ER, et al. Immune response, ciprofloxacin activity, and gender differences after human experimental challenge by two strains of enterotoxigenic *Escherichia coli*. Infect Immun **2007**; 75:252–9.
3. McKenzie R, Bourgeois AL, Frech SA, et al. Transcutaneous immunization with the heat-labile toxin (LT) of enterotoxigenic *Escherichia coli* (ETEC): protective efficacy in a double-blind, placebo-controlled challenge study. Vaccine **2007**; 25:3684–91.
4. Levine MM, Ristaino P, Marley G, et al. Coli surface antigens 1 and 3 of colonization factor antigen II- positive enterotoxigenic *Escherichia coli*: morphology, purification, and immune responses in humans. Infect Immun **1984**; 44:409–20.
5. McConnell M, Hibberd M, Penny M, Scotland S, Cheasty T, Rowe B. Surveys of human enterotoxigenic *Escherichia coli* from three different geographical areas for possible colonization factors. Epidemiol Infect **1991**; 106:477–84.
6. Shaheen HI, Khalil SB, Rao MR, et al. Phenotypic profiles of enterotoxigenic *Escherichia coli* associated with early childhood diarrhea in rural Egypt. J Clin Microbiol **2004**; 42:5588–95.
7. Viboud GI, Jouve MJ, Binsztein N, et al. Prospective cohort study of enterotoxigenic *Escherichia coli* infections in Argentinean children. J Clin Microbiol **1999**; 37:2829–33.
8. Anantha RP, McVeigh AL, Lee LH, et al. Evolutionary and functional relationships of CFA/I and other Class 5 adhesive fimbriae of enterotoxigenic *Escherichia coli*. Infect Immun **2004**; 72:7190–201.
9. Steinsland H, Valentiner-Branth P, Perch M, et al. Enterotoxigenic *Escherichia coli* infections and diarrhea in a cohort of young children in Guinea-Bissau. J Infect Dis **2002**; 186:1740–7.
10. McVeigh A, Fasano A, Scott DA, et al. IS1414, an *Escherichia coli* insertion sequence with a heat-stable enterotoxin gene embedded in a transposase-like gene. Infect Immun **2000**; 68:5710–5.
11. Sjoling A, Wiklund G, Savarino SJ, Cohen DI, Svennerholm AM. Comparative analyses of phenotypic and genotypic methods for detection of enterotoxigenic *Escherichia coli* toxins and colonization Factors. J Clin Microbiol **2007**; 45:3295–301.
12. Evans DG, Evans DJ Jr., Tjoa W. Hemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhea: correlation with colonization factor. Infect Immun **1977**; 18:330–7.
13. Svennerholm AM, Holmgren J, Black R, Levine MM, Merson MM. Serologic differentiation between antitoxin responses to infection with *Vibrio cholerae* and enterotoxin-producing *Escherichia coli*. J Infect Dis **1983**; 147:514–22.
14. Ahren C, Wenneras C, Holmgren J, Svennerholm AM. Intestinal antibody response after oral immunization with a prototype cholera B subunit-colonization factor antigen enterotoxigenic *Escherichia coli* vaccine. Vaccine **1993**; 11:929–34.
15. Levine MM, Nalin DR, Hoover DL, Bergquist EJ, Hornick RB, Young CR. Immunity to enterotoxigenic *Escherichia coli*. Infect Immun **1979**; 23:729–36.
16. Clemens J, Savarino S, Abu-Elyazeed R, et al. Development of pathogenicity-driven definitions of outcomes for a field trial of a killed oral vaccine against enterotoxigenic *Escherichia coli* in Egypt: application of an evidence-based method. J Infect Dis **2004**; 189:2299–307.