Establishment and Validation of Pathogenic CS17+ and CS19+ Enterotoxigenic Escherichia coli Challenge Models in the New World Primate Aotus nancymae

Eric R. Hall,a* Aisling O’Dowd,b,c* Julianne E. Rolenhagen,b,c Nereyda Espinoza,a Gladys Nunez,a Stephen J. Savarino,c,d*

aBacteriology Department, Naval Medical Research Unit No. 6, APO, Lima, Peru
bHenry M. Jackson Foundation for the Advancement of Military Medicine, Bethesda, Maryland, USA
cEnteric Diseases Department, Naval Medical Research Center, Silver Spring, Maryland, USA
dDepartment of Pediatrics, Uniformed Services University of the Health Sciences, Bethesda, Maryland, USA

Eric R. Hall and Aisling O’Dowd contributed equally to this work. Eric R. Hall was the Bacteriology Department head and the principal investigator on the IACUC protocol, and he directly supervised and participated in the experiments and therefore is listed first.

ABSTRACT Enterotoxigenic Escherichia coli (ETEC) is a common cause of diarrheal illness in the military, travelers, and children living in low- to middle-income countries. Increased antibiotic resistance, the absence of a licensed vaccine, and the lack of broadly practical therapeutics perpetuate the significant health and financial burden resulting from ETEC infection. A critical step in the evaluation of vaccines and therapeutics is preclinical screening in a relevant animal disease model that closely replicates human disease. We previously developed a diarrheal model of class 5a colonization factor (CF) CFA/I-expressing ETEC in the New World owl monkey species Aotus nancymae using ETEC strain H10407. In order to broaden the use of the model, we report here on the development of A. nancymae models of ETEC expressing the class 5b CFs CS17 and CS19 with strains LSN03-016011/A and WS0115A, respectively. For both models, we observed diarrheal attack rates of ≥80% after oral inoculation with 5 × 10⁷ CFU of bacteria. These models will aid in assessing the efficacy of future ETEC vaccine candidates and therapeutics.

KEYWORDS enterotoxigenic Escherichia coli, CS17, CS19, vaccine, nonhuman primates

Enterotoxigenic Escherichia coli (ETEC) is a Gram-negative bacteria that causes debilitating diarrheal disease in low- to middle-income countries (1–7). ETEC infection results in long-term sequelae in endemic populations and economic and logistical burdens on military deployed to these regions, as well as on travelers to these regions. These disease consequences and the increase in antibiotic resistance necessitate the development of a broadly effective vaccine and preventative or therapeutic advancement. A primary hindrance to such advancement is circumventing the antigenic heterogeneity of virulence factors expressed by human pathogenic strains (8). Specifically, ETEC expresses various combinations of colonization factors (CF), heat-labile toxin (LT), and/or heat-stable toxins (STp and STh). These toxins lead to the induction of diarrhea after CF-mediated attachment of the bacteria to the mucosa through adhesin subunits (9). The CFs expressed by pathogenic strains range from the well-characterized class 5 fimbriae consisting of a rigid stalk of repeating pilin subunits with a tip-localized adhesin subunit to the fibrillar CS3, afimbrial CS6, and helical CFs such as CS5 and CS7, among many others (10). For vaccine development efforts, preventing this intestinal attachment and interrupting the initial step in ETEC pathogenesis has been a primary focus (11). However, any effective approach must be designed in such a way as to accommodate the broad diversity of pathogenic strains and the CFs they express (12).
The need for such a disease intervention strategy and the increased costs associated with the clinical evaluation of such strategies has led to a renewed interest in an effective preclinical model of disease with which to evaluate therapeutics and vaccines. Small animal models offer a cost-effective method for preliminary screening of candidates and are particularly useful for evaluating the immunogenicity of antigens as well as investigating the pathogenesis of ETEC. However, in order to induce and evaluate diarrheal disease, they often require surgical intervention, such as the rat model (13), the rabbit ileal loop (RIL) (14), and reversible intestinal tie adult rabbit diarrhea (RITARD) (15) models, or they utilize routes and measurable outcomes that lack clinical relevance, such as the intranasal mouse model (16, 17), require antibiotic treatment (18, 19), or have death as the measurable endpoint (20).

To more accurately mimic the diarrheal disease observed in humans, efforts to develop a preclinical model of ETEC diarrhea turned toward nonhuman primates. After initial studies in Old World monkeys failed to reproducibly exhibit a ≥70% attack rate (21), we successfully developed a preclinical model of ETEC infection in the nonhuman primate Aotus nancymae whereby animals orogastrically given 5 × 10^{11} CFU of the CFA/I-positive (CFA/I^{1}) ETEC strain H10407 (LT^{1} STh^{1} Stp^{1}) exhibited diarrhea with an attack rate of 80% (22). Using this model, we demonstrated significant protection following intranasal (i.n.) administration of the CFA/I adhesin-based vaccine, donor strand-complemented CfaE (dscCfaE), and the B subunit of LT (LTB) (22). Relative to small animal models, the orogastric administration, measurable diarrhea, and less invasive nature of this model more precisely mirrors natural ETEC disease in humans and does so in a more closely related species. Therefore, this model may offer a more accurate estimation of the protective efficacy of vaccine candidates or disease prevention provided by therapeutics. However, given the diversity of pathogenic strains and the consequential necessity for any vaccine or therapeutic to be broadly effective, additional models of ETEC disease utilizing strains expressing other CFs are needed.

In the present study, we set out to determine whether strains expressing CFs from a different class 5 family would also induce diarrhea in A. nancymae and thus provide a larger bank of preclinical models available. Specifically, we considered strains producing CS17 and CS19, two closely related CFs belonging to the class 5b family that have been identified in human ETEC (10, 23–26). CS17 consists of the major structural subunit CsβA and the tip adhesin CsβD, while CS19 consists of the major structural subunit CsβA and the tip adhesin CsβD (25). CS17 and CS19 are closely related to CS1, a member of the class 5b family and a CF commonly found in pathogenic isolates (12, 27). Unlike CS1, which is often coexpressed with CS3, CS17 and CS19 are almost always expressed alone, with the former being expressed in almost exclusively LT-only strains (27). These factors support the development of preclinical models of CS17 and CS19 with which to evaluate class 5b-based and LT-based vaccine candidates and therapeutics. We have established a controlled human infection model using the CS17^{1} and CS19^{1} ETEC strains LSN03-016011/A and WS0115A, respectively (28). Challenge in human volunteers with 6 × 10^{9} CFU of LSN03-016011/A resulted in an attack rate of 88%, while 9 × 10^{9} CFU of WS0115A resulted in an attack rate of 44%. Here, we show that diarrhea can also be induced in A. nancymae after challenge with either CS17^{1} ETEC strain LSN03-016011/A or CS19^{1} ETEC strain WS0115A. These models not only allow preclinical evaluation of class 5b-based and LT-based subunit vaccines but also enable assessment of within-CF class cross-protection against ETEC with antigenically similar, but distinct, CFs. Importantly, the current work helps establish a greater bank of diarrhea challenge models with which to preclinically screen various individual or multivalent CF-based vaccine candidates, as well as experimental preventative and therapeutic products.

RESULTS

CS17 challenge model development. A. nancymae orally challenged with 4.9 × 10^{10} or 5.0 × 10^{11} CFU of CS17^{1} ETEC strain LSN03-016011/A exhibited diarrhea in a
Diarrhea after challenge with CS17<sup>+</sup> ETEC, CS19<sup>+</sup> ETEC, or <i>E. coli</i> strains

<table>
<thead>
<tr>
<th>Study</th>
<th>Strain</th>
<th>Challenge dose (CFU)</th>
<th>No. of animals</th>
<th>No. of animals with diarrhea/total no. of animals (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Median no. of days to onset of diarrhea (IQR)&lt;sup&gt;bc&lt;/sup&gt;</th>
<th>Median no. of days diarrhea duration (IQR)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS17, dose finding (expt 1)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>HS</td>
<td>4.3 × 10&lt;sup&gt;11&lt;/sup&gt;</td>
<td>3</td>
<td>1/3 (33)</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>LSN03-016011/A</td>
<td>4.9 × 10&lt;sup&gt;10&lt;/sup&gt;</td>
<td>5</td>
<td>2/5 (40)</td>
<td>1</td>
<td>3 (2–4)</td>
</tr>
<tr>
<td></td>
<td>LSN03-016011/A</td>
<td>5.0 × 10&lt;sup&gt;11&lt;/sup&gt;</td>
<td>5</td>
<td>4/5 (80)</td>
<td>1</td>
<td>7.5 (3.75–9)</td>
</tr>
<tr>
<td>CS17, validation (expt 2)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>HS</td>
<td>4.9 × 10&lt;sup&gt;11&lt;/sup&gt;</td>
<td>5</td>
<td>0/5 (0)</td>
<td>1</td>
<td>7 (4–9.75)</td>
</tr>
<tr>
<td></td>
<td>LSN03-016011/A</td>
<td>5.0 × 10&lt;sup&gt;11&lt;/sup&gt;</td>
<td>10</td>
<td>8/10 (80)</td>
<td>1</td>
<td>8 (4.75–9.75)</td>
</tr>
<tr>
<td>CS19, strain finding (expt 3)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>HS</td>
<td>5.0 × 10&lt;sup&gt;11&lt;/sup&gt;</td>
<td>3</td>
<td>0/3 (0)</td>
<td>1 (1–2)</td>
<td>4 (2–4.5)</td>
</tr>
<tr>
<td></td>
<td>WS0115A</td>
<td>4.8 × 10&lt;sup&gt;11&lt;/sup&gt;</td>
<td>5</td>
<td>5/5 (100)</td>
<td>1</td>
<td>8 (4.75–9.75)</td>
</tr>
<tr>
<td></td>
<td>DS26-1</td>
<td>4.6 × 10&lt;sup&gt;11&lt;/sup&gt;</td>
<td>5</td>
<td>4/5 (80)</td>
<td>1</td>
<td>4 (3–4)</td>
</tr>
<tr>
<td>CS19, validation (expt 4)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>HS</td>
<td>5.0 × 10&lt;sup&gt;11&lt;/sup&gt;</td>
<td>5</td>
<td>0/5 (0)</td>
<td>1</td>
<td>8 (4.75–9.75)</td>
</tr>
<tr>
<td></td>
<td>WS0115A</td>
<td>4.9 × 10&lt;sup&gt;11&lt;/sup&gt;</td>
<td>10</td>
<td>8/10 (80)</td>
<td>1</td>
<td>8 (4.75–9.75)</td>
</tr>
</tbody>
</table>

<sup>a</sup>A diarrheal episode was defined as 2 or more consecutive days of grade 3 or higher stool consistency that begins as the first day of at least 2 consecutive days of diarrhea starting as early as day 1 after challenge and ends when 2 or more days pass without diarrhea.

<sup>b</sup>IQR, interquartile range.

<sup>c</sup>The CS17<sup>+</sup> LSN03-016011/A strain of ETEC (serotype O8:H<sup>+</sup>–, LT<sup>+</sup> STh<sup>+</sup> STp<sup>−</sup>), lot no. PD-7872-44 was used as the challenge strain in experiments 1 and 2 (Cambrex, East Rutherford, NJ).

<sup>d</sup>The CS19<sup>+</sup> ETEC strains WS0115A (serotype O114:H<sup>+</sup>–, LT<sup>+</sup> STh<sup>+</sup> STp<sup>−</sup>) clone H, lot no. 1302 and DS26-01 (O8:H9, LT<sup>+</sup> STh<sup>+</sup> STp<sup>−</sup>) clone G, lot no. 1299 were used as the challenge strains in experiments 3 and 4 (Cambrex).

With clear dose-dependent fashion, yielding attack rates of 40% (2/5) and 80% (4/5), respectively, while 33% (1/3) of animals challenged with nonpathogenic <i>E. coli</i> strain HS had diarrhea (Table 1, experiment 1; Fig. 1A). Diarrheal onset and duration data are shown in Table 1. All test animals shed LSN03-016011/A ETEC by 1 day postchallenge. The median percentages of loose stool days recorded during the observation period for the 5.0 × 10<sup>11</sup> ETEC, 4.9 × 10<sup>10</sup> ETEC, and 4.3 × 10<sup>11</sup> HS groups were 60, 20, and 0, respectively, though there was no significant difference between groups (Fig. 1B). Anti-CS17 IgG and IgA immune responses were observed following challenge with LSN03-016011/A and were consistently higher in the 5.0 × 10<sup>11</sup> dose group compared to the 4.9 × 10<sup>10</sup> dose group. Anti-LT antibodies were not detectable throughout the testing period (Fig. 2A to C).

These data were confirmed in a larger cohort of animals receiving the 5.0 × 10<sup>11</sup> CFU dose of LSN03-016011/A. (Table 1, experiment 2; Fig. 1C). The observed 80% (8/10) diarrheal attack rate was significantly higher than in animals challenged with 4.9 × 10<sup>11</sup> CFU of <i>E. coli</i> strain HS (0/5; 0%) (<i>P</i> < 0.01). All animals challenged with LSN03-016011/A shed ETEC by 2 days postchallenge. The median proportion of days with loose stools for the LSN03-016011/A recipients was significantly higher than animals receiving HS (55% and 0%, respectively) (<i>P</i> < 0.05) (Fig. 1D). Anti-CS17 IgG and IgA and anti-LT(R192G) IgG serum responses mirrored those observed in experiment 1, though at overall lower levels (Fig. 2D to F).

**CS19 challenge model development.** To identify a CS19<sup>+</sup> ETEC strain that would induce a high diarrheal attack rate in <i>A. nancymae</i>, three groups of animals were tested with approximately 5 × 10<sup>11</sup> CFU of CS19<sup>+</sup> ETEC strains WS0115A and DS26-1 and the <i>E. coli</i> strain HS (Table 1, experiment 3). Challenge with WS0115A and DS26-1 resulted in diarrheal attack rates of 100% and 80%, respectively (Fig. 1E). Diarrheal onset and duration data are shown in Table 1, and the proportions of loose stool days are shown in Fig. 1F. All animals challenged with either WS0115A or DS26-1 shed ETEC by 1 day postchallenge. Anti-CS19 IgG and IgA antibody responses were low following CS19<sup>+</sup> ETEC challenge, though they were slightly higher in the WS0115A group compared to the DS26-1 group (Fig. 3A and B). Anti-LT antibodies were low to nondetectable throughout the testing period (Fig. 3C).

Strain WS0115A was validated in a second, larger cohort (Table 1, experiment 4). Animals challenged with 4.9 × 10<sup>11</sup> CFU of WS0115A exhibited a significantly higher diarrheal attack rate of 80% (8/10) than that of animals challenged with 5.0 × 10<sup>11</sup> CFU of <i>E. coli</i> strain HS (0/5; 0%) (<i>P</i> < 0.01). Diarrheal onset and duration data are shown in
FIG 1 Stool characteristics of *A. nancymae* challenged with various doses of CS17+ or CS19+ pathogenic ETEC, or nonpathogenic *E. coli* strain HS. Diarrhea attack rate (A, C, E, and G) and percentage of total loose stool days (B, D, F, and H) are shown. For percentage total loose stool days, data from individual animals are shown as well as the median (bar) for the group. (A and B) Attack rate of diarrhea (A) and percentage of loose stool days (B) in animals given 4.9 × 10^11 or 5.0 × 10^11 CFU of LSN03-016011/A or 4.3 × 10^11 CFU of HS. (C and D) Attack rate of diarrhea (C) and percentage of loose stool days (D) in animals given 5.0 × 10^11 CFU of LSN03-016011/A or 4.9 × 10^11 CFU of HS. (E and F) Attack rate of diarrhea (E) and percentage of loose stool days (F) in animals given 4.8 × 10^11 CFU of WS0115A, 4.6 × 10^11 CFU of DS26-1, or 5.0 × 10^11 CFU of HS. (G and H) Attack rate of diarrhea (G) and percentage of loose stool days (H) in animals given 4.9 × 10^11 CFU of WS0115A or 5.0 × 10^11 CFU of HS.
Table 1, and all animals challenged with WS0115A shed ETEC by 1 day postchallenge (data not shown). The median proportions of days with loose stools for the WS0115A and HS groups were 40% and 10%, respectively (P, 0.005) (Fig. 1H). Anti-CS19 IgG antibody titers against WS0115A were higher in this experiment than in experiment 3 (Fig. 3D to F).

DISCUSSION

In the work presented herein, we developed an ETEC challenge model in the non-human primate Aotus nancymae with the pathogenic CS17+ ETEC strain LSN03-016011/A, demonstrating that oral challenge with 5 x 10^11 CFU resulted in a consistently high attack rate. Importantly, this strain expresses only the LT toxin, allowing for the clear evaluation of the protective efficacy of LT-based vaccine candidates in the future. We also evaluated the ability of two pathogenic CS19+ ETEC strains, WS0115A and DS26-1, to induce diarrhea in the monkey, and demonstrated attack rates of 100%.
and 80% after challenge with approximately $5 \times 10^{11}$ CFU of WS0115A and DS26-1, respectively. Validation of the WS0115A challenge in a second experiment demonstrated an attack rate of 80% after challenge with $5 \times 10^{11}$ CFU WS0115A with the slight difference in attack rates between these two studies, possibly due to the low number of animals used. The studies presented herein were conducted prior to those in the published human studies (28) and were instrumental in the development of the CS17 and CS19 human challenge models.

It is worth reviewing some limitations to these models and the data presented herein. Relatively high doses of inoculum were required to obtain an attack rate suitable for use in evaluating vaccine candidates and therapeutics without increasing sample size. Comparable doses were also required to elicit an attack rate of 80% in the CFA/I-H10407 A. nancyae model (22). High inoculums are also required in many controlled human infection models (CHIM) (29, 30) to reach statistical

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**FIG 3** Serum antibody responses over time in A. nancyae challenged with $4.8 \times 10^{11}$ CFU CS19+ ETEC strain WS0115A or $4.6 \times 10^{11}$ CFU CS19+ ETEC strain DS26-1 or $5.0 \times 10^{11}$ CFU nonpathogenic E. coli strain HS in experiment 3 (A to C) and $4.9 \times 10^{11}$ CFU strain WS0115A or $5.0 \times 10^{11}$ CFU HS in experiment 4 (D to F). (A and D) Anti-CS19 IgG serum responses. (B and E) Anti-CS19 IgA serum responses. (C and F) Anti-LT IgG serum responses. All values are the mean log$_{10}$ titers ± SD. Day of challenge is indicated by a vertical dotted line. The horizontal dotted line denotes the lowest dilution tested.
significance in small subject groups and do not necessarily reflect the amount necessary to cause illness in a natural setting (30, 31). Despite _A. nancymaeae_ being a cost-effective option compared to Old World monkeys or CHIM, relative to small animal models, there are limitations in study size due to cost and study logistics as well as the need to minimize the number of animals exposed to the experimental strains. These restrictions in group size limited the ability to power the study design and prevented a more thorough examination of multiple dose levels for each strain, and as such, doses tested were based on prior experience with the CFA/I<sup>+</sup> H10407 ETEC strain (22). In this study, we’ve expanded beyond the CFA/I<sup>+</sup> H10407 _A. nancymaeae_ model previously presented (22) to include an examination of antibodies elicited through challenge. While anti-CS17 antibody responses to CS17<sup>+</sup> ETEC challenge were consistently robust in the first two experiments, anti-CS19 antibody responses to CS19<sup>+</sup> challenge in experiments 3 and 4 were less so. Further, there were noticeable differences in levels of anti-CS19 antibody responses between the two experiments. The source of these differences is unclear and requires further investigation. Interestingly, anti-LT antibody responses were consistently low across all experiments, despite the presence of CF-specific antibody responses and the reliable induction of diarrhea. The reason for this is not clear; however, we have similarly observed very low anti-LT antibodies in _A. nancymaeae_ exhibiting diarrhea after challenge with CFA/I<sup>+</sup> H10407 (unpublished observations). Future examination of fecal antibody response would be instructive; however, at the time of this study, these assays were not available and current efforts to develop these assays are underway.

ETEC exhibit a high degree of antigenic heterogeneity, particularly in the CFs that are expressed. While studies in adults (12) and children (27) suggest that some of the most prominent CFs include CFA/I and CS1 to CS6, an accurate profile of the CFs presented by circulating pathogenic strains has been difficult to obtain. Further, a large proportion of pathogenic strains have no identified CF (12). This diversity in CFs has hindered the advancement of a broadly protective vaccine, and while there are some vaccine candidates in development (32, 33), none have achieved licensure. ETEC vaccine development efforts by our laboratory have focused on fimbrial adhesins, minor structural subunits of CFs that facilitate intestinal adhesion. Within the class 5 fimbriae, which are phylogenetically organized into three distinct subclasses, 5a (CFA/I, CS4, and CS14), 5b (CS1, CS17, CS19, and PCFO71), and 5c (CS2), the adhesin subunits have greater sequence conservation than the structural subunits that form the fimbrial stalk (25). We hypothesize that this conservation may allow a representative adhesin from each class to provide sufficient protection against other within-class CFs, resulting in a more broadly protective ETEC vaccine. As such, we are developing adhesin subunit vaccine candidates representing each class 5 subclass, and the models presented herein will allow for the evaluation of protection and within-class cross-protection of our class 5b subunit vaccine candidate.

The ability to screen and down-select preventative or therapeutic products prior to evaluation in the more costly controlled human infection models is imperative. In developing the CS17 and CS19 ETEC _A. nancymaeae_ models of disease, we allow for the evaluation of protection and, importantly, cross-protection of vaccines or therapeutic products against two class 5b expressing ETEC strains, including one that is also an LT-only strain of ETEC (LSN03-016011/A). Such models will be highly useful in the down-selection of preclinical products prior to human clinical trials.

**MATERIALS AND METHODS**

**Animal use and welfare.** Studies were reviewed and approved by the Institutional Animal Care and Use Committee at the U.S. Naval Medical Research Unit No. 6 (NAMRU-6), Lima, Peru (protocol number NMRCD 04-1 [NRD-312]) in compliance with all applicable federal regulations governing the protection of animals and research. _Aotus nancymaeae_ was purchased from the Instituto Veterinario de Investigaciones Tropicales y de Altura (IVITA), University of San Marcos, Peru, and randomly assigned to test groups in four separate experiments (Table 2). Animals were housed in the NAMRU-6 Primate Facility, Lima, Peru, fed a standard monkey diet supplemented with fruit, and provided water _ad libitum_. Animals were caged individually beginning 3 days prior to and during the challenge period. Animals

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previously used in ETEC studies or with a baseline reciprocal anti-CS17 (experiments 1 and 2) or anti-CS19 (experiments 3 and 4) IgG titer >300 or which met the diarrhea case definition 3 days prior to challenge were excluded. Weights, temperatures, complete blood counts, and blood chemistry were monitored by the veterinary staff throughout the experiments.

**Challenge inoculum preparations.** Challenge ETEC strains used in the four studies described herein are listed in Table 2. cGMP manufactured vials of each strain were manufactured under contract by Cambrex (East Rutherford, NJ). The CS17\(^+\) LSN03-016011/A strain of ETEC, serotype O8:H\(^-\), LT\(^+\)STh\(^-\)STp\(^-\) (lot no. PD-7872-44) was used as the challenge strain in experiments 1 and 2. The CS19\(^+\) W50115A and DS26-01 strains of ETEC, serotypes O114:H\(^-\), LT\(^+\)STh\(^-\)STp\(^-\) (clone H, lot no. 1302 and DS26-01 O8:H9, LT\(^+\)STh\(^-\)STp\(^-\)) and O8:H9, LT\(^+\)STh\(^-\)STp\(^-\) (clone G, lot no. 1299), respectively, were used as the challenge strains in experiments 3 and 4. The HS strain of *E. coli* is a nonpathogenic nontypeable *E. coli* strain isolated from the stool of a healthy adult and was included as a negative control (34). All challenge inocula were prepared by harvesting bacteria after incubation on colonization factor antigen (CFA) agar with bile salts for 14 to 28 h and then harvesting bacteria after incubation on colonization factor antigen (CFA) agar with bile salts for 14 to 28 h. Plates with between 30- and 300-well isolated colonies were selected for counting. The number of viable organisms was calculated, and the dose was calculated by back titration. The resulting most probable numbers of bacteria in prechallenge and postchallenge preparations were averaged to give greater accuracy to the number of bacteria administered to animals (22, 35).

**Challenge model development and disease assessment.** Animals (n = 3 to 10/group) were orally challenged on study day 0 with various doses of CS17\(^+\) or CS19\(^+\) ETEC strains or the control strain HS (Table 1) as previously described (22) and observed twice daily for 10 days for the development of diarrhea according to the following stool grading system: grade 1, hard (normal); grade 2, soft (normal); grade 3, thick liquid (diarrhea); grade 4, opaque-water (diarrhea), and grade 5, clear/watery (diarrhea). A diarrheal episode began on the first of two or more consecutive days of grade 3 or higher stool consistency and ended the day prior to two or more consecutive days of only grade 2 or lower stool consistency. The percentage of total loose stool days was calculated as the number of loose stool days of grade 3 or higher was observed divided by the total number of observation days multiplied by 100. Fecal excretion was monitored daily for 5 days after challenge as previously described using CF-specific antisera (22). Animals were treated with enrofloxacin (5 mg·kg\(^{-1}\)) administered intramuscularly (i.m.) once daily for 5 days after the 10-day observation period.

**Analysis of antibodies in serum samples by ELISA.** Sera were tested by enzyme-linked immunosorbent assay (ELISA) for the presence of antibodies against CS17, CS19, and LT (with LT(R192G) as the reagent) using standard techniques previously described (22). Binding of IgG and IgA antibodies were detected with rabbit anti-A. *nancyae* IgG-horseradish peroxidase (HRP) conjugate (lot no. 101H0804; Lampire Biological Laboratories, Pipersville, PA) and rabbit anti-A. *nancyae* IgA-HRP conjugate (lot no. 043063740; Lampire Biological Laboratories), respectively. The HRP-specific substrate used was ortho-phenylenediamine (Sigma, St. Louis, MO). The serum samples were serially diluted, and the endpoint titers were assigned as the interpolated dilutions of the samples, giving an absorbance value at 450 nm of 0.4 optical density units above the background. The antibody titer ascribed to each sample represented the geometric mean of duplicate determinations. Serum samples with undetectable titers (i.e., reciprocal endpoint titer of <5) were assigned a value of 2.5 for computational purposes.

### Table 2: *A. nancyae* demographics

<table>
<thead>
<tr>
<th>Study</th>
<th>Strain</th>
<th>Challenge dose (CFU)</th>
<th>Demographic variable</th>
<th>No. of animals</th>
<th>No. of males/ no. of females</th>
<th>Mean age (mo [SD])(^a)</th>
<th>Mean wt (grams [SD])(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS17, dose finding (expt 1)(^b)</td>
<td>HS</td>
<td>4.3 × 10(^{11})</td>
<td>3</td>
<td>0/3</td>
<td>31.0 (0.3)</td>
<td>1,330 (78.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LSN03-016011/A</td>
<td>4.9 × 10(^{10})</td>
<td>5</td>
<td>3/2</td>
<td>25.9 (1.6)</td>
<td>996 (63.48)</td>
<td></td>
</tr>
<tr>
<td>CS17, validation (expt 2)(^b)</td>
<td>HS</td>
<td>4.9 × 10(^{11})</td>
<td>5</td>
<td>3/2</td>
<td>22.0 (1.8)</td>
<td>960 (148)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LSN03-016011/A</td>
<td>5.0 × 10(^{11})</td>
<td>5</td>
<td>3/2</td>
<td>23.1 (2.6)</td>
<td>1,002 (187.8)</td>
<td></td>
</tr>
<tr>
<td>CS19, strain finding (expt 3)(^c)</td>
<td>HS</td>
<td>5.0 × 10(^{11})</td>
<td>3</td>
<td>2/1</td>
<td>20.3 (0.3)</td>
<td>1,093 (110.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>W50115A</td>
<td>4.8 × 10(^{11})</td>
<td>5</td>
<td>2/3</td>
<td>19.3 (0.03)</td>
<td>974 (128.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DS26-1</td>
<td>4.6 × 10(^{11})</td>
<td>5</td>
<td>1/4</td>
<td>21.8 (1.8)</td>
<td>936 (83.85)</td>
<td></td>
</tr>
<tr>
<td>CS19, validation (expt 4)(^c)</td>
<td>HS</td>
<td>5.0 × 10(^{11})</td>
<td>5</td>
<td>1/4</td>
<td>20.4 (4.5)</td>
<td>786 (55.95)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>W50115A</td>
<td>4.9 × 10(^{11})</td>
<td>10</td>
<td>7/3</td>
<td>21.3 (7.0)</td>
<td>889 (207.1)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Age and weight data collected on day 3.
\(^b\)The CS17 \(^+\) LSN03-016011/A strain of ETEC (serotype O8:H\(^-\), LT\(^+\)STh\(^-\)STp\(^-\)), lot no. PD-7872-44 was used as the challenge strain in experiments 1 and 2 (Cambrex, East Rutherford, NJ).
\(^c\)The CS19 \(^+\) ETEC strains W50115A (serotype O114:H\(^-\), LT\(^+\)STh\(^-\)STp\(^-\)) clone H, lot no. 1302 and DS26-01 (O8:H9, LT\(^+\)STh\(^-\)STp\(^-\)) clone G, lot no. 1299 were used as the challenge strains in experiments 3 and 4 (Cambrex).
Statistical analyses. For all experiments, the proportion of animals experiencing diarrhea in each test group was compared to that in the control group with a Fisher’s exact test. Frequency analyses were not adjusted for multiple comparisons. A Fisher’s exact test with a 5% two-sided significance level was used to determine that, for the validation experiments (experiments 2 and 4), a sample size of 5 animals receiving the HS strain and 10 animals receiving the ETEC strain (either LSN03-016011/A or W50115A) will have 76% power to detect a significant difference if the HS attack rate is approximately 0% and the ETEC strain attack rate is 80%. The percentage of days with loose stool for each animal was compared between groups using a Kruskal-Wallis test followed by Dunn’s multiple-comparison test or a Mann-Whitney U test. A P value of <0.05 was considered significant, and tests were interpreted in a two-tailed fashion. GraphPad Prism version 6.07 for Windows (GraphPad Software, San Diego, CA) was used for all statistical analyses.

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REFERENCES


