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REVIEW

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A roadmap for enterotoxigenic *Escherichia coli* vaccine development based on volunteer challenge studies

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ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of travelers' diarrhea and of diarrhea among young children in developing countries. Experimental challenge studies in adult volunteers have played a pivotal role in establishing ETEC as an enteric pathogen, elucidating its pathogenesis by identifying specific virulence attributes, characterizing the human immune response to clinical and sub-clinical ETEC infection and assessing preliminarily the clinical acceptability, immunogenicity and efficacy of prototype ETEC vaccines. This review provides a historical perspective of experimental challenge studies with ETEC. It summarizes pioneering early studies carried out by investigators at the University of Maryland School of Medicine to show how those studies provided key information that influenced the directions taken by many research groups to develop vaccines to prevent ETEC. In addition, key experimental challenge studies undertaken at other institutions will also be cited.

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Introduction

Enterotoxigenic *Escherichia coli* (ETEC), a diarrheagenic pathotype of *E. coli*, is currently recognized as a major cause of dehydrating diarrheal illness among human infants, toddlers and pre-school children in developing countries,[1–3] as well as a prominent cause of travelers' diarrhea when persons from industrialized countries travel to developing countries.[4,5] While ETEC is not a major pathogen associated with sporadic diarrheal illness in industrialized countries, occasional outbreaks of ETEC diarrhea have been described in the U.S. A. and other industrialized countries.[6-8] As the acceptance of ETEC as a diarrheal pathogen among young children (and to a lesser extent adults) in developing countries and among adult travelers became increasingly widespread, researchers began to develop vaccines to prevent ETEC illness in those target populations.

Over the decades, experimental challenge studies in adult volunteers have played a pivotal role in establishing ETEC as an enteric pathogen, elucidating its pathogenesis by identifying specific virulence attributes, characterizing the human immune response to clinical ETEC infection and assessing preliminarily the safety, clinical acceptability, immunogenicity and efficacy of prototype ETEC vaccines. This review will provide a historical perspective of experimental challenge studies with ETEC. The focus will be on pioneering early studies carried out by investigators at the University of Maryland School of Medicine to show how those studies provided key information that influenced the directions taken by many research groups to develop vaccines to prevent ETEC. In addition, key experimental challenge studies undertaken at other institutions will also be cited.

Initial incrimination of ETEC as a human pathogen

In the 1960s few bacterial pathogens were unequivocally accepted as etiologic agents of diarrhea. The few included *Vibrio cholerae* O1, *Shigella*, non-typhoidal *Salmonella*, and enteropathogenic *E. coli* of classical infant serotypes. However, since the 1950s it had been recognized that cell free culture supernatants of *V. cholerae* O1 were able to cause intestinal secretion within ligated rabbit ileal loops identified by prominent dilatation and fluid accumulation within the loops.[9] It is against this background that a team of American investigators from the Johns Hopkins University School of Medicine working in Calcutta (Kolkata) with their Indian colleagues from the Calcutta School of Tropical Medicine and the Calcutta Infectious Disease Hospital were studying non-*Vibrio* cholera in adult patients in the late 1960s. Patients with this syndrome presented with clinical dehydration and exhibited copious purging but the duration and total volume of purging was significantly curtailed compared to true cholera patients with *V. cholerae* O1 infection; clinically, only a minority of these adult patients needed intravenous fluids to maintain hydration compared to confirmed cholera cases. When intestinal intubation was performed, proximal intestinal fluid from the non-*Vibrio* cholera patients yielded pure cultures of *E. coli* at a titer of 10^6–10^8 colony forming units (CFU) per milliliter.[10] Sack et al showed that bacteria-free culture supernatants from cultures of *E. coli* isolated from the small intestine of four patients with non-*Vibrio* cholera syndrome led to distention of isolated rabbit ileal loops indicating the presence of enterotoxin(s).[11] The culture-free supernatant from *E. coli* strain 334 was found to be non-dialyzable, precipitated in 40% ammonium sulfate,

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diminished after heating to 80°C for 30 minutes,[11] and was eliminated by exposure to 100°C for 2 minutes.

A hallmark volunteer challenge study documents that ETEC can cause diarrhea in healthy U.S. adults

The elegant early reports of Sack et al and others from the Kolkata team provided strong suspicion that ETEC were the likely etiologic agents of non-*Vibrio* cholera in adults living in a cholera-endemic area.[10–12] However, it was not yet clear if these organisms were causes of the diarrheal illness experienced by industrialized country persons traveling to developing countries,[13] or of infant diarrhea in developing regions where cholera was or was not endemic. During this period a major military conflict was ongoing in Vietnam and U.S. military personnel who were deployed there were experiencing a high incidence of traveler's diarrhea during the early weeks and months of their deployments. Accordingly, the U.S. Department of the Army supported experimental challenge studies in volunteers carried out by investigators from the University of Maryland School of Medicine (led by Herbert L. DuPont) and the Walter Reed Army Institute of Research (led by Samuel B. Formal).[14] These investigators tested the ability of two ETEC strains, B2C (serotype O6:H16) and B7A (serotype O148:H28), isolated from U.S. soldiers in Vietnam with non-bloody watery diarrhea, for their ability to elicit diarrhea when fed to healthy U.S. adults.[14] Bacterial cell-free supernatants of cultures of each of these two strains, as well as of porcine ETEC strain 263 (serotype O8:K87,K88a,b:H19) known to cause diarrhea in piglets,[15] elicited fluid accumulation when inoculated into isolated rabbit ileal loops.[14] Another group of volunteers ingested a normal flora *E. coli* strain, HS, that was isolated from a healthy U.S. adult without diarrhea.[14] Since inoculation of isolated rabbit ileal loops with whole bacterial cultures or with bacteria-free culture supernatants of strain HS failed to cause fluid accumulation in rabbit ileal loops, *E. coli* HS was considered to be a putative normal human intestinal flora strain.

Groups of five volunteers each ingested inocula containing either 10^8 or 10^{10} CFU of one of these *E. coli* strains, yielding the results summarized in Table 1. The bacteria were administered as a suspension in 45 ml of milk to fasting volunteers who received no pretreatment with buffer to neutralize gastric acid. The volunteers in this one study were healthy adults incarcerated in a penal institution.[14] They underwent informed consent procedures similar to the consent procedures used in current clinical investigations. The volunteers in all subsequent ETEC challenge studies in this review were free-living community volunteers, in particular college students.

Both these human ETEC strains originally isolated from U.S. soldiers with non-dysenteric travelers' diarrhea caused severe watery diarrhea in 60%-80% of the volunteers who ingested the higher inoculum without buffer. In contrast, the normal flora *E. coli* strain that exhibited no enterotoxic activity in rabbit ileal loops caused neither mild nor severe diarrhea in the volunteers who ingested either 10^8 or 10^{10} CFU. Another notable observation was that a virulent porcine ETEC strain known to cause severe watery diarrhea in piglets

Table 1. Human ETEC, porcine ETEC and human normal flora non-ETEC strains fed to healthy U.S. volunteers at a dose of 10^8 CFU or 10^{10} CFU[a]

E. coli strain	Source of the strain	Cell-free culture supernatant caused fluid accumulation in rabbit ileal loops	No .of volunteers who ingested the strain at each inoculum level	Attack rate for diarrhea following ingestion of 10^8 CFU by healthy U.S. volunteers		Attack rate for diarrhea following ingestion of 10^{10} CFU by healthy U.S. volunteers		Positive stool cultures following ingestion of 10^8 CFU	Positive stool cultures following ingestion of 10^{10} CFU
				Mild[b]	Severe[c]	Mild[b]	Severe[c]		
B2C	U.S. soldier with travelers' diarrhea	Yes	5	2/5 (40%)	0/5 (0%)	0/5 (0%)	3/5 (60%)	5/5 (100%)	5/5 (100%)
B7A	U.S. soldier with travelers' diarrhea	Yes	5	1/5 (20%)	0/5 (0%)	0/5 (0%)	4/5 (80%)	4/5 (80%)	5/5 (100%)
263	Porcine strain that causes severe diarrhea in piglets	Yes	5	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	5/5 (100%)	4/5 (80%)
HS	Normal flora strain from a healthy US adult	No	5	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	4/5 (80%)	5/5 (100%)

[a]The data in this table were adapted from DuPont HL et al 1971[14].
[b]Mild diarrhea was defined as ≥ 3 watery less stools/24-hour period.
[c]Severe diarrhea was defined as ≥ 5 watery stools/24-hour period for two consecutive days.

leading to high mortality was as well tolerated in human volunteers as the normal flora *E. coli* strain HS. This observation added to evidence that was beginning to accumulate in animal studies indicating that virulence factors other than enterotoxin conferred species specificity to the ability of ETEC to cause diarrheal illness.[16] The report of DuPont et al led to widespread acceptance of certain ETEC as human diarrheal pathogens.[14] DuPont et al also reported volunteer challenge studies that established enteroinvasive *E. coli* as another pathotype of *E. coli* that can cause diarrhea in human adults.[14]

Insights from early veterinary and animal model studies with ETEC derived from pigs, calves and humans

Sack et al emphasized the similarity of the heat-labile enterotoxin activity of ETEC to that of cholera toxin.[11] However, in that same late 1960s period, as veterinary investigators were demonstrating that ETEC were etiologic agents of "colibacillosis" diarrheal illness in neonatal piglets, they clearly described the existence of two distinct types of enterotoxin in porcine strains, one heat-labile and non dialyzable (i.e., having a moderate or high molecular weight) but the other heat-stable and dialyzable (implying a small molecular weight).[17] They noted that some porcine ETEC produced both enterotoxins, while others appeared to produce only one or the other. Veterinary investigators were also the first to show that enterotoxic activity could be transferred to a non-pathogenic *E. coli* strain by transfer of a plasmid presumed to encode the porcine enterotoxins.[18] Veterinary investigators also showed the key role of fimbrial colonization factors (CFs) such as K88 in the pathogenesis of porcine ETEC diarrhea and reported that these CFs that mediate attachment to intestinal mucosa were also encoded on transferrable plasmids.[16]

Other investigators focusing on ETEC isolated from humans with non-dysenteric diarrheal illness devised ways to differentiate the presence of the heat-stable ETEC enterotoxin (ST) from the heat-labile toxin (LT). Besides molecular weight and resistance to heating, the time onset of fluid accumulation in isolated rabbit ileal loops following inoculation with culture supernatants provided a clear-cut albeit complex way to differentiate the two enterotoxin activities. Intestinal secretion from ST peaked at 4–6 hours after inoculation, whereas LT fluid accumulation peaked at 18 hours and was not evident at 4 hours.[19]

Two detection methods for LT and ST that fostered initiation of epidemiologic studies

The early practical breakthroughs for differentiating the presence of LT versus ST in culture supernatants came in the mid-1970s with: 1) the discovery that Y-1 adrenal cells and Chinese hamster ovary (CHO) cells in tissue culture monolayers underwent a striking change in their morphology following exposure to LT consequent to the toxin activating adenylate cyclase leading to an intracellular accumulation of cyclic AMP;[20–22] and 2) the development of the infant (suckling) mouse assay.[23] These early phenotypic assays were not simple, economical or high-throughput compared to modern nucleic acid-based diagnostics for identifying *E. coli* producing LT, ST or both. Nevertheless, they were sufficiently robust and practical so that it was possible to undertake small and moderate size epidemiologic studies to begin to define the burden of ETEC disease. Before these assays were available, earlier small studies had indicated that most ETEC isolates from cases of diarrhea produced both LT and ST or only LT (based on fluid accumulation kinetics in rabbit ileal loops). With early application of these new assays, particularly the infant mouse assay, it became evident that isolates from some cases of watery diarrhea produced only ST.[6,24,25]

Volunteers challenged with ST-only ETEC

The notion that ETEC strains that produce exclusively ST, without LT, might be able to cause diarrhea in humans was met with skepticism by some investigators.[26] Indeed, the overall evidence indicting ST-only ETEC as a pathogen was not compelling. Accordingly, Levine et al at the Center for Vaccine Development of the University of Maryland School of Medicine (CVD) carried out an experimental challenge study in healthy adult community volunteers (in particular college students) to determine if a well characterized (for the time) ST-only strain would induce diarrhea in volunteers.[27] ETEC strain 214–4 was isolated from a U.S. traveler to Mexico who developed typical travelers' diarrhea with watery stools, abdominal cramping, nausea and fever. Culture supernatants of 214–4 were positive in the infant mouse assay for ST but negative in the Y-1 adrenal cell assay for LT.[27]

In step-wise, dose-escalating fashion, small groups of volunteers ingested 10^6, 10^8 or 10^{10} CFU of ST-only strain 214–4 after fasting for 2.5 hours prior to challenge. In these initial studies no $NaHCO_3$ pretreatment was given and the ETEC inoculum was administered in 45 ml of milk.

A clear dose-response was observed (Table 2). Those who ingested 10^6 did not develop clinical illness but three of four volunteers shed the challenge strain and two mounted four-fold rises in serum antibody to 214–4 O antigen. Recipients of 10^8 CFU exhibited a clinical attack rate of 60% and manifested mild, short-lived (one day) clinical illness after a two-day incubation period. Volunteers who ingested 10^{10} CFU showed an 80% attack rate for diarrhea that was markedly more severe (up to 1.5 liters in total volume) than in recipients of lower inocula. The incubation period was one day shorter and the illness lasted longer (2–3 days) in the recipients of 10^{10} CFU. In subsequent studies when 10^9 CFU of ETEC strain 214–4 were administered with $NaHCO_3$ pretreatment to neutralize gastric acid, a 90% attack rate of diarrheal illness ensued among the challenged volunteers.

When the challenge study with 214–4 was performed in the mid-1970s, it was not yet recognized that there exist two distinct types of ST, most often referred as human ST (STh) and porcine ST (STp).[28] Strain 214–4 is now known to encode STp and to express coli surface antigen 6 (CS6), a member of the CFA/IV family of colonization factors. The morphology of CS6 is not known; it is neither fimbrial nor fibrillar.[29]

Table 2. Clinical, bacteriologic shedding and immunologic response of groups of healthy adult community volunteers who ingested 10^6, 10^8 or 10^{10} colony forming units (CFU) of ETEC strain 214–4 that produces only ST[a]

Inoculum ingested (CFU)	No .of volunteers who ingested the strain	Attack rate for clinical illness (%)				Mean incubation (hours)	Positive stool cultures	≥ 4-fold rise over baseline in O antibody titer	≥ 4-fold rise in anti-LT antitoxin titer
		Any	Diarrhea[b]	Vomiting	Fever[c]				
10^6	4	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)	–	3/4 (75%)	2/4 (50%)	0/4 (0%)
10^8	5	3/5 (60%)	2/5 (20%)	1/5 (0%)	0/5 (0%)	43	5/5 (100%)	4/5 (80%)	0/5 (0%)
10^{10}	5	4/5 (80%)	4/5 (80%)	0/5 (0%)	2/5 (40%)	27	5/5 (100%)	4/5 (80%)	0/5 (0%)

[a]Data in this summary were adapted from Levine et al.[27] Volunteers who had been fasting for 2.5 hours prior to challenge ingested the ETEC inoculum in 45 ml of milk with no NaHCO3 pretreatment.
[b]In this early challenge study diarrhea was defined as ≥ 2 loose stools within 24 hours or at least one loose stool > 200 ml in volume
[c]Fever was defined as an oral temperature ≥ 100°F

Milestone and landmark on the roadmap

This volunteer challenge study established unequivocally the capacity of ST-only ETEC to cause diarrheal illness and prophesied that an effective vaccine against ETEC would have to confer protection against ST-only and LT/ST ETEC phenotypes.[27] Subsequent large epidemiologic studies have documented that the vast majority of the attributable ETEC disease burden is due to pathogens that encode ST, either alone or with LT.[1,2]

Assessing infection-derived immunity based on homologous and heterologous re-challenges

The next hallmark volunteer challenge study undertaken at CVD had several aims, the foremost of which was to address the question of whether an initial clinical episode of diarrhea due to an ETEC strain producing both LT and ST (i.e., LT/ST strain) would confer protection against re-challenge with the homologous LT/ST ETEC strain.[30] To answer this first question, ETEC strain B7A was selected since it produces both LT and ST and DuPont et al had shown some years before that B7A caused diarrheal illness in volunteers.[14] At the time of this study B7A was known not to express CFA/I or CFA/II but did express type I somatic pili.[14] At the time B7A could not be tested for CFA/IV as this family of major CF antigens had not yet been discovered.[31-33]

The college students and other healthy community adult volunteers, were fasted for 90 minutes prior to and after challenge. They ingested 240 ml of water containing 2 g of $NaHCO_3$ to buffer gastric acid one minute before ingesting the inoculum of ETEC strain B7A suspended in 45 ml of phosphate-buffered saline. Volunteers were given an inoculum of 10^6 or 10^8 CFU to identify a dose, as administered, that would induce a clinical attack rate for diarrheal illness of 60–70%. The 10^6 CFU inoculum elicited diarrheal illness in 3 of 6 volunteers (50%), while the 10^8 CFU inoculum led to diarrheal illness in 7 of 11 (64%) of volunteers. The clinical syndrome in the volunteers was typical of adult travelers' diarrhea with 17–18% also manifesting fever. All volunteers in both dose groups excreted the challenge organism.

Eight of the 10 volunteers in the initial challenge who developed diarrhea agreed to return nine weeks later to participate in a homologous re-challenge study in which these eight volunteers plus 12 naïve control volunteers ingested 10^8 CFU of B7A. Upon re-challenge only 1 of 8 "veterans" (12.5%) developed diarrhea versus 7 of 12 naive volunteers (58.3%) who ingested B7A for the first time ($p = 0.05$) (Table 3), demonstrating that an initial clinical infection confers 79% protection against ETEC diarrhea upon subsequent re-exposure to the homologous pathogen.[30] Although significant protection against clinical illness was observed, the re-challenged volunteers shed B7A organisms in their stools with the same frequency as the naïve controls. This implied that whatever immune response was mediating protection against clinical illness did not involve a bactericidal mechanism as was observed among cholera veterans who were protected upon re-challenge and had drastically reduced excretion of *Vibrio cholerae* O1 amongst the re-challenged veterans even when re-challenged three years after their initial challenge.[34,35]

Since ST is a small peptide that is not immunogenic in the course of natural infection, the mechanism of protection had to be mediated by immune responses other than anti-ST. One hypothesis proffered was that intestinal secretory immune

Table 3. Protection conferred by a prior clinical diarrheal infection due to experimental challenge with enterotoxigenic *Escherichia coli* strain B7A (O148:H28) against subsequent challenge nine weeks later with 10^8 colony forming units of strain B7A versus naïve volunteers[a].

Study group	Diarrheal illness[b] attack rate (%)	Protective efficacy	Subjects with positive stool cultures
B7A "veterans"	1/8[c] (12.5%)	–	8/8
Naïve controls	7/12[d] (58.3%)	79%	12/12

[a]Data in this table are adapted from Levine et al 1979[30].
All stools were graded and weighed. Grade 1 was a fully formed fecal bolus; grade 2 was a soft stool that did not take the shape of the stool cup; grade 3 was a thick liquid stool; grade 4 was an opaque watery stool; grade 5 was a rice water stool.
In this early study diarrhea was defined as at least two loose (grade 3–5) stools within 24 hours or one large loose stool ≥ 200 grams.
(Note – in later studies diarrhea as an efficacy end point following challenge was defined as the passage of ≥2 loose stools (grade 3–5) over a 48-h period totaling at least 200 grams, or a single loose stool ≥300 grams).
[c]vs [d], $p = 0.05$.

responses to an unknown colonization factor antigen might have mediated protection by preventing attachment to intestinal mucosa. Interestingly, it was shown one decade later that B7A encodes the CS6 antigen of the CFA/IV family.[32,36]

More than three decades later, investigators at the Johns Hopkins School of Public Health corroborated the early CVD report of the protection conferred by a single initial challenge with ETEC followed by re-challenge with the homologous strain.[37,38] Ten veterans who had experienced clinical diarrhea several weeks previously were re-challenged, along with 10 naive volunteers, with 10^7 CFU of ETEC strain H10407 (O78: H11, LT/ST, CFA/I) administered with buffer. The attack rate for diarrhea was 9/10 in the naives versus only 1/10 re-challenged veterans, showing 89% protection. Both naives and veterans shed the challenge strain but quantitative counts in the latter showed a circa 2-log reduction in the number of H10407 per gram of stool.

Milestone and landmark on the roadmap

The early CVD volunteer challenge study established that an initial clinical ETEC infection with an LT/ST strain confers ~79% protection against the occurrence of diarrhea upon subsequent exposure to the homologous ETEC strain, which causes a moderate clinical attack rate in naïve controls.

Early studies with LT-only ETEC

Another aim of this set of early volunteer challenge studies at CVD was to establish whether an epidemiologically incriminated LT-only ETEC strain, E2528-C1 (serotype O25:NM) identified as the cause of a large outbreak of diarrheal illness aboard a cruise ship,[39] would cause diarrhea in adult U.S. volunteers. A small study established that naïve volunteers did develop diarrheal illness following ingestion of LT-only strain E2528-C1.[30] Diarrheal illness was observed after a notably short incubation period (9.5 –22.5 hours) and was mild and short-lived; nevertheless it was evident.

A third aim was to see whether veterans of B7A (serotype O148:H28) diarrheal illness who mounted robust anti-LT responses would be prevented from developing diarrhea when challenged with LT-only ETEC strain E2528-C1 (serotype O25: NM).[30] Four volunteers who recovered from diarrhea while serving as controls in the homologous re-challenge study of B7A and who agreed to ingest LT-only strain E2528-C1 were re-challenged 10 weeks later with 10^9 CFU of E2528-C1, while six naïve volunteers served as controls. The small numbers in each group provided little statistical power to detect a meaningful difference other than if complete protection had been observed. In fact, diarrhea occurred in 3 of 4 of the B7A veterans versus in 2 of 6 naïve controls. Thus, no evidence indicating heterologous protection was observed. The four B7A veterans who participated in the heterologous re-challenge had all exhibited ≥ 4-fold rises in serum LT neutralizing antitoxin reciprocal titer following their initial challenge (<4 to 256; <4 to 128; 8 to 32; 32 to 128). However, by the time of the heterologous re-challenge 10 weeks later, the serum LT antitoxin reciprocal titers in these veterans had fallen markedly to 64, 4, 4 and 8. Since both B7A and E2528-C1 produce LT and volunteers challenged with B7A had initially mounted robust anti-LT responses following their first challenge, it was initially concluded that anti-LT, as elicited by a wild type infection, was insufficient to confer protection against challenge with a heterologous serotype expressing only LT. However, in this study intestinal antitoxin titers had not been measured to help interpret the lack of protection, recognizing that serum antitoxin may not reflect SIgA antitoxin titers in the small intestine. Finally, it was subsequently determined that E2528-C1 expresses CS6, just like B7A. So another more modern interpretation would be that anti-CS6 responses if they occurred were also not protective. Anti-CS6 titers in serum or intestinal fluid were not measured.

Milestone and landmark on the roadmap

An epidemiologically well-incriminated LT-only strain was able to induce diarrhea in healthy adults. This suggested that a subset of LT-only strains are pathogenic for humans.

Role of colonization factors in the pathogenesis of ETEC diarrhea and in mediating protection

Veterinary studies

Studies by veterinary investigators in piglets and calves established unequivocally that plasmid-encoded fimbrial antigens played a critical role in the pathogenesis of ETEC diarrhea by attaching the bacteria to receptors on intestinal mucosa of animals of certain susceptible genotypes.[40] The veterinary studies also blazed a path for immunization based on preventing intestinal colonization of infant animals by immunizing pregnant sows and cows parenterally with purified K88, K99 or 987P fimbrial CF antigens.[41-44] Such immunization led to antibodies in the colostrum of immunized sows and cows and neonatal piglets and calves suckled on immunized sows and cows were significantly protected against challenge with a lethal dose of virulent ETEC expressing the homologous CF, while piglets and calves suckled on control sows and cows developed severe diarrhea. It was concluded that the key protective moiety present in the colostrum consumed by the piglets and calves suckled on immunized mothers was specific anti-K88, anti-K99 or anti-987 antibody.

Studies in humans

Identification of the major CFs of human ETEC pathogens

The early studies of veterinary researchers in piglets led investigators intending to develop vaccines to prevent human ETEC disease to evaluate an array of approaches to immunize actively with experimental products intended to stimulate immune responses (particularly intestinal SIgA) against the main CFs encoded by human ETEC, and against other ETEC surface or exported antigens. In groundbreaking microbiological studies, Evans et al discovered in ETEC strain H10407, isolated from a patient with severe diarrhea in East Pakistan (now Bangladesh), a fimbrial antigen that they initially called Colonization Factor,[45] and subsequently referred to

as colonization factor antigen I (CFA/I). The expression of CFA/I by H10407 shared many characteristics with the expression of K88 fimbriae in piglet strains. Evans et al showed that the expression of CFA/I and the ability to cause diarrhea in an infant rabbit model required the presence of a ~ 60 MD plasmid.[45] A derivative strain that had lost the ~60 MD plasmid, strain H10407P, did not express CFA/I and was no longer able to cause diarrhea in infants rabbits, despite retaining the ability to express LT. Subsequent analyses showed that the CFA/I plasmid lost by H10407P also encoded STh. Volunteer challenge experiments carried out by Satterwhite et al and Evans et al at the University of Texas Medical School at Houston showed that H10407 caused overt diarrheal illness in healthy adult community volunteers, while H10407P caused no adverse clinical consequences.[46,47] Evans et al reported that ETEC strains expressing CFA/I hemagglutinated human group A erythrocytes in the presence of mannose, thereby distinguishing the surface antigen from type 1 somatic pili which mediate agglutination of guinea pig erythrocytes but that hemagglutination is inhibited by the presence of mannose.

Evans et al later reported that certain ETEC strains of serotypes O6:H16 and O8:H9 expressed a distinct new CF that they called CFA/II and that it could be detected by the strain's exhibiting mannose-resistant hemagglutination of bovine erythrocytes but not of human blood group A erythrocytes, as did CFA/I isolates.[48] Sera from rabbits immunized with CFA/II-positive strain PB176 (O6:H16, biotype A) were absorbed with variant strain PB176-P that had lost the property of MRHA of bovine erythrocytes to prepare an antibody against an antigen of ETEC strain PB176.[48]

Other investigators reported that CFA/II in fact constituted a family of antigens. Cravioto et al described a "component 3" antigen that was common to ETEC of O6:H16 strains of biotypes A, B and C. In addition, O6:H16 ETEC of biotype A also expressed component 1.[49] In contrast, O6:H16 ETEC of biotypes B and C expressed component 2 antigen, in addition to component 3. Smyth showed that CFA/II-positive ETEC occurred among a variety of O serogroups and referred to the common antigen (component 3) as "CS3" (coli surface antigen 3), while component 1 was designated CS1 and component 2 as CS2; this nomenclature became widely adopted.[50] All CFA/II strains express CS3, sometimes alone and otherwise in conjunction with CS1 or CS2.

The morphology of CS1 and CS2 was shown by electron microscopy to consist of rigid fimbriae circa 6–7 nm diameter, resembling CFA/I. Early attempts by various investigators to visualize the morphology of intact CS3 were not successful.[51,52] However, Levine et al eventually showed that CS3 consists of wiry, flexible, thin fibrillae 2–3 nm in diameter.[53]

Levine et al challenged 14 volunteers with an inoculum of 5×10^8 viable organisms of E24377A, an O139:H28 LT/ST strain of ETEC that expresses CS1 and CS3, to verify the pathogenicity of this strain and to measure the serum and mucosal IgA immune responses to purified CS1 and CS3 antigens elicited by wild type infection. As summarized in Table 4, nine of the 14 community volunteers developed diarrheal illness typical of travelers' diarrhea. Six of the nine clinically ill volunteers exhibited significant rises to both CS1 and CS3, as did one of five non-ill volunteers. Paired jejunal fluids from eight volunteers were tested for rises in intestinal antibody to purified CS1 and CS3 antigens. A baseline and a single convalescent specimen of jejunal fluid were collected 7 days after ingestion of the ETEC and two of five persons with diarrhea but none of three without diarrhea manifested four-fold or greater rises in SIgA antibody to the fimbriae.

The CFA/IV family of ETEC CF antigens was next described, originally as "E8775" antigens.[54] All ETEC within this family express CS6, either alone or in combination with CS4 or CS5. CFA/I, CFA/II family and CFA/IV family are now commonly referred to as the major CFs. CS4 and CS5 consist of rigid fimbriae ~ 5–7 nm in diameter, while a morphology has not been ascribed to CS6.

Milestone and landmark on the roadmap. Data from the volunteer challenge with CFA/II strain E24377A demonstrated that both CS1 and CS3 antigens are expressed *in vivo* in the course of ETEC infection of persons from a non-endemic area and that most of those who developed diarrheal illness mounted both serum IgG and intestinal IgA responses to the fibrillar CS3 antigen common to all CFA/II strains, as well as to CS1.

Table 4. Clinical and immunologic response of 14 healthy adult community volunteers who ingested 5×10^8 colony forming units (CFU) of ETEC strain E24377A (O139:H28) that expresses CS1 and CS3 colonization factors and produces LT and ST.

				No. with ≥ 4-fold rise over baseline in serum anti-CS3 or anti-CS1 antibody titer (%)		≥ 4-fold rise over baseline in intestinal fluid IgA antibody to CS3 and CS1 (%)[b]	
Clinical response to challenge	No .of volunteers who ingested the strain	No. with positive culture (%)	Mean total diarrheal stool (g[a])	Purified CS3	Purified CS1	Purified CS3	Purified CS1
Diarrhea	9	9/9 (100%)	787 (384–1139)	6/9 (67%)	6/9 (67%)	2/5 (40%)	2/5 (40%)
No diarrhea	5	5/5 (100%)	1001 (517–1429)	1/5 (20%)	1/5 (20%)	0/3 (0%)	0/3 (100%)

[a]Data in this summary were adapted from Levine et al[53].
[b]Jejunal were collected only at baseline and at 7 days post-challenge but at no later time points. Data shown are only for volunteers with paired specimens. Jejunal fluids were gently centrifuged to remove particulate matter; the supernatants were saved, SIgA concentrations were measured by radial immunodiffusion, and the fluids were lyophilized. Jejunal fluids were reconstituted to 20 mg of SIgA per 100 ml before testing for specific antibody. A mixture of purified CS1 and CS3 in a ratio of 1:20 was applied as antigen to microtiter plates. Twofold dilutions (1:4 to 1:128) of jejunal fluid were dispensed to wells, and an enzyme-linked immunosorbent assay was performed.[53].

Identification of minor CFs among ETEC isolated from humans with diarrheal illness

Considerable evidence from epidemiologic studies and from experimental challenges in volunteers indicated that the seven major CFS including CFA/I, the CFA/II family (CS1, CS2, CS3) and the CFA/IV family (CS4, CS5, CS6) constitute key virulence properties and potentially important antigens to be included in an ETEC vaccine based on stimulating protection by preventing ETEC from attaching to receptors on enterocytes in the proximal small intestine. This strategy triggered many studies to ascertain the proportion of ETEC from cases of travelers' diarrhea and from endemic pediatric diarrhea that encode or express these seven CFs. Those studies suggest that only circa 50–70% of ETEC isolates from putative cases of ETEC diarrhea carry the major CFs. Thus, many investigators undertook to identify additional novel CFs expressed by human ETEC that did not encode or express the major CFs and that might be potential CF antigens to be added to a CF-based ETEC vaccine to broaden protection.

Approximately two dozen minor CFs have been described.[55] Whereas these minor CFs share certain properties with major CFs and typically show adherence to tissue culture cells *in vitro*, the actual epidemiologic data supporting their role as pathogens have been relatively modest and sparse. Some minor CFs such as CS7 encoded by LT-only ETEC have been incriminated based on data from a few cohort or case/control studies of infant diarrhea,[56,57] and on results from one volunteer challenge study.[58] Minor CFs CS7, CS14, CS17 and CS19 have elicited considerable interest.[59] CS7 and CS17 may represent virulence attributes of a subset of LT-only ETEC, separating them from other LT-only ETEC that are apparently non-pathogenic and may have been derived from LT/ST strains by gene loss.[48,60-62]

A key volunteer challenge study addressing the question of the pathogenicity of CS17 LT-only ETEC was carried out by McKenzie et al of the Johns Hopkins School of Public Health who administered to healthy adult community volunteers inocula of 7×10^8 or 6×10^9 CFU (with buffer) of O8:H9 ETEC strain LSN03-016016/A that expresses CS17 and LT but does not express ST nor major CFs.[58] This LT-only strain expressing CS17 as the only recognized CF encoded by that strain caused diarrhea in 60% of volunteers who ingested the lower inoculum and in 88% who ingested the higher inoculum. The majority of challenged volunteers mounted serum IgA or IgG antibody responses against CS17 and LT (Table 5).

Milestone and landmark on the roadmap. The Mckenzie et al[58] challenge study confirmed the earlier study of Levine et al[30] showing that some LT-only strains can cause unequivocal diarrheal illness following experimental challenge of U.S. adult volunteers. The study of McKenzie et al implicated CS17 as an adhesion factor *in vivo*.[58] Disappointingly, no volunteer challenge studies have been reported wherein, in parallel, groups of randomly allocated volunteers were fed a wild type CF-positive ETEC strain or its isogenic deletion mutant engineered to disable expression of the entire CF or of the adhesive tip protein, while leaving intact other virulence attributes.[63]

Experiences with early vaccine candidates

Parenteral type 1 somatic pili vaccine studies at CVD

Duguid, who coined the term fimbriae (Latin for fibers),[64] and Brinton,[65] who used the term pili (Latin for hairs), first brought the attention of scientists to the thinner non-flagella protein appendages that emanate from many bacteria. While conjugal or sex pili allow transfer of DNA from one bacterium to another, other categories of organelles were also described, of which type 1 somatic pili (also called common pili) that confer the properties of hemagglutination of guinea pig erythrocytes and adhesion to tissue culture cells were the earliest to be characterized.[64,66,67] These hemagglutinating and cell adhering properties were ablated in the presence of D-mannose (i.e., mannose-sensitive hemagglutination [MSHA]).[66,67] Type 1 somatic pili were believed to attach normal flora *E. coli* to the mucosa of the human colon.[67] When ETEC were discovered and shortly thereafter CFA/I and CFA/II were found to be expressed by some ETEC isolates, with the former conferring mannose-resistant hemagglutination of human group A erythrocytes and the latter manifesting mannose-resistant hemagglutination of bovine erythrocytes, preliminary surveys of ETEC and normal flora *E. coli* were undertaken.[68,69] These early reports noted that type 1 somatic pili were found with equal frequency among ETEC and normal flora *E.coli*, whereas CFA/I and CFA/II were detected only among ETEC isolates.

It is not well recognized that the first ETEC vaccine to be tested that was intended to prevent diarrheal illness in humans was a parenterally-administered product consisting of purified type 1 somatic pili from ETEC strain H10407; this vaccine was prepared by Charles L. Brinton of the University

Table 5. Clinical and immunologic response of small groups of healthy adult community volunteers who ingested 10^8 or 10^9 colony forming units (CFU) of ETEC strain LSN03–016011/A that produces only LT and expresses CS17 in the absence of expression of major CFs[a]

Inoculum ingested (CFU)[b]	No .of volunteers who ingested the strain	Attack rate for diarrhea (%)	Mean total diarrheal stool (g[a])	> 4-fold rise over baseline in serum anti-CS17 antibody titer (%)		> 4-fold rise over badeline in serum anti-LT antitoxin titer (%)	
				IgG	IgA	IgG	IgA
10^6	5	3/5 (60%)	787 (384–1139)	3/5 (60%)	5/5 (100%)	4/5 (80%)	4/5 (80%)
10^9	8	7/8 (88%)	1001 (517–1429)	6/8 (75%)	6/8 (75%)	8/8 (100%)	8/8 (100%)

[a]Data in this summary were adapted from Mckenzie et al[58].
[b]Volunteers who had been fasting for 90 minutes prior to challenge ingested the ETEC inoculum suspended in $NaHCO_3$ buffer following $NaHCO_3$ pretreatment to neutralize gastric acid.
[c]Diarrhea was defined as > 2 loose stools within 48 hours that totaled > 200 g or at least one loose stool > 300 g in weight.

of Pittsburgh. Brinton and team were developing pilus-based vaccines to prevent gonorrhea and ETEC diarrhea. Type 1 somatic pili were originally intended to be a potential vaccine antigen within a multivalent pili-based combination vaccine that was expected to protect against ETEC strains that did not express CFA/I and CFA/II, the only known CFs among human ETEC at the time. Brinton, hypothesized that type 1 somatic pili represented a general attachment factor for ETEC, as well as for normal flora, and opined that antibody to these surface organelles, which inhibited attachment to tissue culture cells *in vitro*, would presumably also do so in the human small intestine.[67] Brinton argued that inclusion of type 1 somatic pili in a multivalent vaccine, along with CFA/I and CFA/II, could broaden the protection conferred by the ETEC vaccine. On the other hand, since normal flora *E. coli* flora are important for healthy human gut physiology and they express type 1 somatic pili, it was important to verify that eliciting immune responses against these organelles did not adversely affect the normal intestinal physiology of humans.

ETEC strain H10407 (O78:H11, LT/ST), which expresses CFA/I, an adhesin found only among ETEC strains, also expresses type 1 somatic pili. By differential growth characteristics and purification methods, Brinton's team was able to purify both type 1 somatic pili and mannose-resistant hemagglutinin (CFA/I) from H10407. Brinton prepared two different formulations of purified type 1 somatic pili for vaccine trials, one for parenteral and the other for oral administration.[69] One Institutional Review Board ethics committee objected to the administration of type 1 somatic pili as an oral vaccine because of safety concerns over potentially disrupting the functions of normal flora *E. coli*. Therefore, clinical trials commenced with administering type 1 somatic pili by the intramuscular route on the assumption that this was the safer route for initial studies. At the behest of the U.S. Department of Defense, CVD initiated studies to assess the clinical tolerability and immunogenicity of parenterally-administered type 1 somatic pili from ETEC strain H10407, followed by multiple small challenge studies to evaluate preliminarily whether there was evidence of vaccine efficacy (Table 6).[70]

The type 1 somatic pili vaccine was given to a total of 100 volunteers in dose-escalation groups who received a single intramuscular dose of either 45, 90, 180, 450, 900 or 1800 mcg. Fifteen individuals received a booster dose 28 days later of 1800 mcg. All single-dose injections were well-tolerated. However, 6 of 15 individuals who received the 1800 mcg booster dose developed local reactions (heat, induration or erythema) at the injection site. The pili vaccine did not affect intestinal transit time or gut absorptive capacity, nor did the vaccine alter the prevalence of normal flora *E. coli* expressing type 1 somatic pili.[70] Recipients of 900 or 1800 mcg doses had significantly higher serum antibody responses on day 28 than recipients of 45 or 90 mcg doses. Importantly, none of the type 1 somatic pili vaccinees mounted serum antibody responses to purified CFA/I, attesting to the purity of the pili. However, O antibody seroconversions were observed in 3 of 11 vaccinees who got 45, 90 or 900 mcg doses and in all 10 recipients of 1800 mcg doses. Notably, following challenge with wild type ETEC strain H10407, control volunteers who

Table 6. Clinical and bacteriologic response of healthy Maryland adults vaccinated with type 1 somatic pili from purified from enterotoxigenic *E. coli* strain H10407 administered parenterally as a priming and booster dose and of unimmunized control volunteers following challenge with different dose levels of virulent homologous enterotoxigenic *E. coli* strain H10407 (O78:H11, LT/ST) or heterologous virulent strain B7A (O148:H28, LT/ST).

Challenge strain inoculum (colony forming units)	Vaccination regimen		Study group	Diarrheal illness attack rate (%)	Vaccine efficacy	p-value	Mean diarrheal stool volume per ill volunteer (range, liters)	Positive stool cultures
	Day 0 prime	Day 28 boost						
H10407 ($5x10^8$)			Controls	7/7 (100%)	–	0.04	4.0 (1.4–9.9)	7/7
	1800 mcg	1800 mcg	Vaccinees	2/6 (33%)	67%		3.9 (1.4–6.4)	6/6
H10407 ($5x10^8$)			Controls	7/8 (88%)	–	0.24	3.0 (0.5–8.6)	8/8
	900 mcg	450 mcg	Vaccinees	3/6 (50%)	43%		4.3 (0.9–10.6)	6/6
H10407 ($5x10^7$)			Controls	3/11 (27%)	–	0.23	1.2 (0.4–1.5)	11/11
	900 mcg	450 mcg	Vaccinees	3/4 (75%)	0%		0.7 (0.3-0.8)	4/4
B7A ($1x10^{10}$)			Controls	4/6 (67%)	–	1.00	0.6 (0.3–1.0)	6/6
	900 mcg	450 mcg	Vaccinees	5/8 (63%)	6%		0.9 (0.3–2.3)	8/8
A338C5 ($1x10^9$)			Controls	5/8 (62%)	–		0.6 (0.1–1.5)	3/8
	1800 mcg	450 mcg	Vaccinees	8/13 (62%)	0%	1.00	0.5 (0.1–1.1)	5/13

Data from challenges with H10407, B7A and A338C5 are adapted from Levine,[71] and Levine et al.[70,72]

developed diarrheal illness did not mount rises in serum or intestinal fluid antibody titer to type 1 somatic pili, although most did show significant rises to CFA/I and all showed significant rises in titer of serum IgG and intestinal IgA O antibody.

The first volunteer challenge study involved six vaccinees who one month earlier had received a booster inoculation with 1800 mcg of vaccine, along with seven unimmunized control subjects. When these study participants ingested 5×10^8 CFU of ETEC strain H10407, all seven challenged control volunteers developed diarrhea versus only 2 of 6 vaccinees (p = 0.021, 2-tail Fisher's Exact test), i.e., 67% vaccine efficacy (Table 6). One of the vaccinees who failed to be protected had voluminous diarrhea (6.4 liters); the maximal stool volume among controls was a purge of 9.9 liters. All six vaccinees and control subjects shed ETEC H10407 in their stools.

This initial small challenge study generated some optimism to explore further whether immune responses to parenterally-administered type 1 somatic pili might be able to confer protection against ETEC. However, before proceeding further, the two-dose immunization schedule had to be modified so that local adverse reactions would not occur. Further clinical testing showed that administering an initial intramuscular priming dose of 900 mcg followed one month later by a booster with either 180 mcg or 450 mcg did not elicit local reactions and by day 28 post-booster the serologic responses achieved were comparable to those that had been seen following an 1800 mcg booster. Accordingly, three additional small challenge studies were undertaken.

In the next study, subjects immunized with the non-reactogenic 900 mcg prime/450 mcg booster immunization schedule were challenged with 5×10^8 CFU of ETEC H10407. Thus, the only difference in the design of this second study was the lower dosage levels of vaccine administered, while the challenge inoculum of H10407 was identical. Diarrhea occurred in 7 of 8 controls (88%) and in 3 of 6 vaccinees (50%), showing 43% vaccine efficacy (p = 0.24) (Table 6). Several vaccinees and controls purged more than 5.0 liters. With the small sample sizes in this study and the modest vaccine efficacy, there was insufficient power to detect a significant difference.

Because of the cholera-like purging that occurred in some control volunteers when the challenge inoculum of H10407 was 5×10^8 CFU, in the next challenge study subjects vaccinated with the non-reactogenic immunization schedule and controls were challenged with an inoculum that was reduced by one log to 5×10^7 CFU (Table 6). This smaller inoculum dropped the attack rate for diarrhea among control to only 3 of 11 (27.3%) and the volume of diarrhea similarly plummeted, as no control passed more than 1.5 liters of diarrhea. The diarrheal illness attack rate in vaccinees was 3 of 6 (50%), showing no vaccine efficacy.

It became obvious that additional efficacy trials with H10407 would not provide a clear answer as to whether antibody to type 1 somatic pili could confer protection against challenge with ETEC. The anti-O78 responses in recipients of high doses of type 1 somatic pili vaccine were a confounder. Moreover, H10407 expresses CFA/I and the type 1 pili vaccine was meant to protect against ETEC strains that lacked known ETEC CFs such as CFA/I or CFA/II. Therefore, two final challenge studies were designed to rule out the effect of O78 antibody as a confounding variable and to match or deliberately mis-match the antigenic similarity between the type 1 pili of the challenge strain and H10407 type 1 somatic pili in the vaccine. Thus, for these last two studies ETEC strains B7A (O148:H28, LT/ST) and A338C5 (O27:H7, ST-only) were carefully selected because these both lack CFA/I or CFA/II. The type 1 somatic pili of B7A are only very distantly related to those of H10407, whereas the type 1 somatic pili of A338C5 are antigenically identical to those of H10407.

Following administration of type 1 somatic pili vaccine according to the well tolerated prime/boost immunization schedule, vaccine recipients and controls were challenged with wild type strain B7A. This challenge was not expected to show protection and indeed there was none, as 5 of 8 type 1 somatic pili vaccinees developed diarrhea (62% attack rate) along with 4 of 6 controls (67%).

In the final study in this series, a cohort of 13 volunteers immunized with an 1800 mcg priming dose followed by a 450 mcg booster dose of type 1 somatic pili and a group of eight control volunteers were challenged with 10^9 CFU of ETEC strain A338C5. Since this was a critical study designed to demonstrate whether or not immune responses to type 1 somatic pili could prevent diarrheal infection due to an ETEC strain of a heterologous O:H serotype that expresses antigenically identical type 1 somatic pili, it was important to document the immune responses of the vaccinated subjects. All 13 vaccinees mounted strong rises in titers of IgG antibody to type 1 somatic pili in serum. Moreover, during the immunization period, jejunal fluid was collected from the 13 vaccinees pre-immunization, 11 days after the booster dose of vaccine and again just prior to challenge (one month after receipt of the booster vaccination) to monitor titers of intestinal SIgA antibody to purified type 1 somatic pili antigen. Twelve of the 13 vaccinees manifested ≥ four-fold rises in SIgA anti-pili antibody. It is not surprising that parenteral immunization with pili was so successful in eliciting significant rises in SIgA anti-pili antibody in jejunal fluid if one assumes that these subjects were all immunologically primed from exposure to type 1 pili expressed by the normal flora *E. coli* in their large intestine. Swedish investigators had previously reported that administration of parenteral whole-cell cholera vaccine to immunologically-primed lactating Pakistani women led to increases in SIgA anti-vibrio antibodies in breast milk, whereas such SIgA responses did not occur in unprimed lactating Swedish women following parenteral cholera vaccination.[73]

Despite the SIgA anti-pili antibody in the small intestine of Maryland volunteers vaccinated parenterally with type 1 somatic pili vaccine, following challenge with ETEC strain A338C5 (O27:H7, LT-/ST+), no protection was observed. Diarrhea occurred in 8 of 13 vaccinees (62%) and 5 of 8 controls (63%), thus 0% vaccine efficacy (Table 6).

Milestone and landmark on the roadmap

These early volunteer challenge studies led to the conclusion that parenterally-administered type 1 somatic pili vaccine

should not be a priority for further clinical development. These early evaluations of this first proposed human vaccine candidate to prevent ETEC diarrhea allowed the experimental challenge model with several different wild type ETEC strains to mature. ETEC strain H10407 was an arduous challenge strain and volunteers had to be closely monitored. Clinical illness in controls given 5×10^8 CFU of H10407 experienced high attack rates of diarrhea that was sometimes cholera-like in quality and volume. However, lowering the inoculum of H10407 by one log to diminish the severity of diarrhea in controls also lowered the attack rate, thereby requiring larger sample sizes. Nevertheless, experimental challenge studies appeared to be a practical way to assess preliminarily the efficacy conferred by ETEC vaccine candidates that could confer a moderately high level of efficacy. Going forward, CVD investigators preferred to explore the use of oral vaccines that included immunogenic virulence factors found exclusively in ETEC, such as CFA/I and CFA/II, rather than an attachment factor (type 1 somatic pili) also expressed by normal flora *E. coli*, as with the latter there would always be a worry about long-term safety. The subsequent studies of candidate vaccines at CVD reflected these conclusions.

Oral purified fimbriae vaccines at CVD

Studies in rabbits had shown that application of purified CFA/II fimbriae to the mucosa of rabbits having exteriorized Thiry-Vella intestinal loops with their own blood supply demonstrated that SIgA anti-fimbrial antibody responses could be elicited but the dose of CFA/II fimbriae required was rather high (2.0 mg) in order to be immunogenic.[72] Based on these results in rabbits, Levine et al immunized 10 healthy adult community volunteers with 2.0 mg of purified CFA/II orally twice weekly for four weeks.[74] To suppress gastric acid secretion and to protect the fimbrial protein against gastric juice during transit through the stomach, the fimbriae were administered in a suspension with 2.0 g of $NaHCO_3$.[74] Only 2 of 10 volunteers developed significant (≥ 4-fold) rises in SIgA or serum IgG anti-CFA/II antibody. Eight vaccinees from this cohort were challenged along with nine control volunteers. ETEC diarrhea was observed in 3 of 8 vaccinees (37.5%) and 6 of 9 (66.7%) of controls, indicating 43.8% vaccine efficacy.[74] With the small numbers in each group this difference in attack rate was not significant ($p = 0.35$).

Despite the administration of cimetidine and $NaHCO_3$ buffer in an attempt to protect the CFA/II fimbrial protein, it was considered possible that the fimbriae were being adversely affected by proteolytic enzymes during passage through the human stomach. Indeed, Schmidt et al showed that even at neutral pH gastric contents adversely affected fimbrial protein.[75] Accordingly, Levine et al performed a proof-of-principle study in healthy community volunteers in which three 5.0 mg doses of CFA/II fimbriae vaccine (given on days 0, 14 and 28) were administered directly into the duodenum via intestinal tube, thereby bypassing the stomach. Administered in this way that bypassed the stomach, the CFA/II fimbrial vaccine stimulated significant rises in anti-CFA/II antibody in four of five vaccinees.[74]

Milestone and landmark on the roadmap

These studies with purified CFA/II fimbriae at CVD indicated that large amounts of fimbrial protein had to be administered and protected from the gastric environment in order to elicit credible seroconversion of SIgA anti-CFA/II antibody. This made the delivery of purified fimbriae logistically difficult and diminished enthusiasm in that approach.

Oral purified fimbriae vaccines at the University of Texas at Houston

Evans et al studied the immunogenicity of a 1:1 combination of purified CFA/I and CFA/II fimbriae administered to 11 healthy adult volunteers orally in milk.[76] The immunization regimen consisted of administering 1 mg doses each of CFA/I and CFA/II in combination daily for four days followed by 2 mg each of CFA/I and CFA/II given in combination on days 11 and 17 of the regimen. Consequently, these orally immunized subjects each received a total of 8 mg of CFA/I and 8 mg of CFA/II. Eleven other volunteers received placebo given according to the same regimen. The serum anti-CFA/I and anti-CFA/II responses were abysmal. Only 3 of 11 oral vaccinees mounted a ≥ 4-fold rise in antibody to CF antigen. One vaccinee responded to both antigens, one to CFA/I and another to CFA/II. However, one of 11 placebo recipients also mounted a ≥ 4-fold rise to CFA/I antigen. All 11 vaccinees and the 11 placebo recipients were challenged with virulent LT/ST ETEC strain H10407 (serotype O78:H11, LT/ST) expressing CFA/I or strain H1765 (O6:H16, LT/ST) expressing CFA/II. Diarrhea occurred in 4 of 5 (80%) of vaccinees and in 2 of 5 (40%) placebo recipients challenged with H10407 and in 5 of 6 (83%) vaccinees and 5 of 6 placebo recipients challenged with ETEC strain H1765, showing no hint of protection.

Circa two years after challenge with ETEC, 11 volunteers who developed diarrhea were invited to return to be immunized orally with a single 1.0 mg dose of CFA/I administered in PBS after the volunteers had ingested $NaHCO_3$ to neutralize gastric acid. Among these 11 subjects, eight exhibited ≥ 4-fold rises in serum anti-CFA/I antibody. Regrettably, the design of this sub-study did not allow one to dissect the relative importance of immunologic priming versus protecting the CF antigen with prior $NaHCO_3$ buffer.

In a final study in this series, Evans et al immunized eight healthy adults subcutaneously with a single 50 mcg dose of purified CFA/I, as a parenteral priming dose, and then boosted these eight subjects with two spaced (one week apart) oral doses (0.5 mg each) of CFA/I antigen administered orally with $NaHCO_3$ buffer. Four of the eight vaccinees exhibited significant rises in intestinal IgA anti-CFA/I following the oral booster dose. Following challenge of these eight vaccinees with ETEC strain H10407 (LT/ST, CFA/I), diarrhea was observed in four vaccinees. Three of the four subjects who did not develop diarrhea following challenge were ones who manifested a significant rise in intestinal IgA anti-CFA/I titer following the oral booster.[76]

CVD studies with purified CFA in biodegradable polylactide/polyglycolide microspheres

Tacket et al carried out a feasibility study of an enteral ETEC vaccine prototype consisting of CFA/II containing two component antigens, CS1 and CS3, encapsulated in biodegradable polymer microspheres (BPM).[77] Ten healthy adult community volunteers swallowed intestinal tubes on days 0, 7, 14 and 28. Following the collection of jejunal fluid samples, 1 mg of CFA/II in BPM was administered via the intestinal tube. Volunteers kept a diary of sign and symptoms after each dose. Secretory IgA in jejunal fluids, serum antibody responses and circulating antibody-secreting cells (ASC) to CFA/II antigens were measured before and after vaccination. The vaccine was well tolerated. Five of ten volunteers developed IgA anti-CFA/II antibody secreting cells (ASCs) by seven days after the last dose of vaccine. Five of ten vaccinees developed rises in jejunal fluid SIgA anti-CFA/II. Circa 8 weeks after the first dose of vaccine, 10 vaccinees and 10 unvaccinated control volunteers were challenged with 10^9 CFU of virulent ETEC strain E24377A (O139:H28; LT/ST; CS1,CS3). Ten of 10 controls and seven of 10 vaccinees developed diarrhea (30% vaccine efficacy; $p = 0.11$).

Milestone and landmark on the roadmap

Disappointingly, the biodegradable polymer microspheres were unable to elicit strong anti-CF antibody responses or to elicit a credible level of protection.

Inactivated ETEC vaccine at the University of Texas at Houston

Evans et al abandoned the concept of purified CF antigen as an ETEC vaccine and devised an innovative way to deliver CF antigens by administering inactivated fimbriated whole-cell ETEC bacteria. Their oral vaccine consisted of intact H10407 (O78:H11, LT/ST, CFA/I) bacterial cells that were treated with the potent DNA endonuclease colicin E2 to render them incapable of proliferating but otherwise leaving intact the surface antigens of the bacteria including CF fimbriae. After neutralizing gastric acid with $NaHCO_3$ pretreatment, healthy adult volunteers were given two oral doses spaced one month apart, of vaccine (containing 3×10^{10} bacteria) or placebo. Serum and intestinal fluid specimens from the proximal small intestine were collected at baseline and one month after the first and second doses of vaccine or placebo. The vaccine was well tolerated. None of the nine placebo recipients exhibited rises in IgA intestinal antibody to purified CFA/I antigen, whereas five of the 10 vaccinated volunteers mounted ≥ 4-fold rises in intestinal IgA anti-CFA/I titer and four other vaccinees exhibited lesser rises.[78] Approximately 7–8 weeks after ingestion of the booster dose of vaccine or placebo, the 19 volunteers were challenged by ingesting, after $NaHCO_3$ pretreatment, 5×10^9 CFU of virulent ETEC strain H10407 (O78:H11;LT/ST, CFA/I). The attack rate for diarrheal illness was 8 of 9 control volunteers (89%) versus 2 of 10 vaccinees (20%), indicating 78% vaccine efficacy ($p < 0.01$, 2-tail Fisher's exact test).[78]

Evans et al next examined whether the colicin E2-inactivated H10407 bacterial whole-cell oral vaccine would protect against challenge with a virulent ETEC expressing CFA/I, LT and ST but of an O:H serotype (O63:H-) heterologous from the H10407 vaccine strain (O78:H11) or a virulent LT/ST ETEC strain, heterologous in serotype (O6:H16) and CF type (CFA/II) compared to the vaccine strain.[79] Twenty-two healthy adult volunteers were given two spaced doses (one month apart) of colicin-inactivated H10407 bacterial vaccine cells, after pre-treatment with $NaHCO_3$ buffer. Eight vaccinees were challenged with 5×10^9 CFU of an O63:H- LT/ST CFA/I strain 6–7 weeks after the second dose of vaccine; only 2 of 8 vaccinees developed diarrhea (25% attack rate). Eight other vaccinees were challenged with the O6:H16 LT/ST CFA/II strain and only 2 of eight developed diarrhea (25% attack rate). However, in neither of these two heterologous challenges was there a placebo or unvaccinated control group to compare the attack rate that would ensue with these challenge strains in unvaccinated persons. Thus, although Evans et al claimed to have achieved 75% protection with the colicin-inactivated vaccine against serotype-heterologous wild type ETEC expressing CFA/I or CFA/II, this claim cannot be substantiated because of the absence of unvaccinated controls exposed to the identical experimental challenge inocula.[79]

Formalin-inactivated ETEC vaccine at CVD

To explore the safety, immunogenicity and efficacy of a simple inactivated whole-cell fimbriated ETEC vaccine, CVD investigators administered formalin-inactivated ETEC to healthy adult community volunteers. The vaccine was prepared by inactivating *E. coli* strain E1392-75-2A, an O6:H16 biotype A strain that expresses CS1 and CS3 fimbriae but does not elaborate either LT or ST. The formalin-inactivated vaccine was prepared at the Pilot Bioproduction Facility of the Walter Reed Army Institute of Research at Forest Glen, MD. Electron microscopic photomicrographs documented that the bacterial cells in the vaccine were fimbriated.[80] Three doses of formalin-inactivated E1392-75-2A vaccine administered with $NaHCO_3$ were given two weeks apart to nine community volunteers. Four of the nine exhibited significant (≥ 4-fold) rises in intestinal SIgA anti-CFA/II antibody and two of nine had significant rises in serum anti-CFA/II antibody. A small challenge study was carried out in which four vaccinees and 10 controls were challenged with fully enterotoxigenic ETEC strain E24377A (O139:H28) which is heterologous in O:H serotype from the inactivated vaccine (O6:H16) but expressed the identical CS1 and CS3 fimbriae. Diarrhea occurred in two of four vaccinees (50% attack rate) and in six of ten controls (60% attack rate). Although this small study was admittedly under-powered, there was no evidence of protection.

Milestone and landmark on the roadmap

This early inactivated fimbriated whole cell vaccine was deemed poorly immunogenic and non-protective, so further work on this approach was abandoned by CVD investigators in favor of other oral vaccine strategies.

Gothenburg University/Scandinavian Biopharma ETVAX

Investigators at the University of Gothenburg have been actively developing an oral ETEC vaccine based on a mix of inactivated ETEC strains expressing selected CFs, in combination with a toxoid to stimulate antitoxin to neutralize LT. A prototype oral cholera vaccine that was the precursor to the licensed Dukoral cholera vaccine, consisting of inactivated *Vibrio cholerae* bacteria combined with the B (binding) subunit of cholera toxin purified by chemical means from the enzymatically active A subunit, elicited moderate protection for circa 4 months against diarrhea caused by LT-expressing ETEC among Bangladeshi adults in a randomized, placebo-controlled field trial.[81] That oral cholera vaccine also conferred upon Finnish travelers to Morocco moderate protection against diarrhea caused by LT-expressing ETEC in a small randomized controlled field trial.[82] Results from these early trials suggested that mucosal SIgA antitoxin stimulated by cholera toxin B subunit provided some degree of cross protection against diarrhea caused by LT-producing ETEC. The Swedish investigators thereupon embarked on development of an oral ETEC vaccine by combining inactivated fimbriated ETEC strains with the B subunit of cholera toxin. A first generation vaccine consisting of cholera toxin B subunit combined with five formalin-inactivated ETEC strains expressing CFA/I, CS1, CS2+CS3, CS4 and CS5, respectively, was immunogenic in Swedish adults and Egyptian and Bangladeshi infants and toddlers but did not confer significant protection in a field trial in Egyptian children and did not protect young adult U.S. travelers from diarrhea caused by ETEC expressing antigens contained in the vaccine.[83]

The Gothenburg investigators thereupon undertook to improve the vaccine. The second generation vaccine called ETVAX contains four formalin-inactivated ETEC strains that hyper-express CFA/I, CS3, CS5 and CS6, in combination with a hybrid CTB-LTB subunit protein that preferentially elicits anti-LT.[84] In some clinical trials ETVAX has also been co-administered with a double mutant LT adjuvant (dMLT).[85] While ETVAX is under clinical evaluation in field studies in children in developing countries and in adult travelers, heretofore, no volunteer challenge studies have been undertaken to assess the efficacy of ETVAX.

Attenuated ETEC strains as live oral vaccines

Live fimbriated, toxin-negative ETEC strain as a prototype live oral vaccine at CVD

Investigators at CVD carried out a dose-escalating safety/immunogenicity study in which sequential small groups of healthy adult community volunteers ingested (after pretreatment with $NaHCO_3$) a single dose of 10^9, 10^{10}, or 6×10^{10} CFU of prototype vaccine strain E1392-75-2A, an O6:H16 serotype that expresses CS1 and CS3 but does not express LT or ST.[72,74,86] This non-toxigenic strain expressing CS1 and CS3 proved to be highly immunogenic when delivered as a prototype live oral vaccine, particularly with respect to the stimulation of SIgA anti-CFA/II antibodies in intestinal fluid. A dose response was observed, with the strongest immune responses recorded in recipients of the highest dose level ($6x10^{10}$ CFU). At this dose level, specimens of jejunal fluid collected from 13 of 15 vaccinees (86.7%) showed that their proximal small intestine was colonized with the live vaccine.[86] Of the 19 vaccinated subjects, paired pre- and post-vaccination intestinal fluids were obtained from 14 individuals.[74] Significant (≥ 4-fold) rises in SIgA antibody to CFA/II antigen were detected in 11 of the 14, including all six participants who ingested a single dose containing 6×10^{10} CFU.

The one drawback to the prototype highly immunogenic live oral ETEC vaccine was that at the highly immunogenic dose of ≥ 10^{10} CFU, 2 of 15 vaccinees (13.3%) developed diarrhea. In one vaccinee the diarrhea was very mild (four loose stools totaling 243 ml), while the other vaccinee had moderate diarrhea and passed nine loose stools over three days totaling 1197 ml.[86] Levine et al likened the diarrhea observed in this small proportion of subjects given a high dose of the prototype vaccine to what was described when veterinary researchers administered a K88-positive non-toxigenic strain of porcine ETEC to piglets.[16] Mild diarrhea was observed in approximately one-third of the piglets whose proximal small intestine was heavily colonized with the non-toxigenic but adhering strain. In contrast, piglets fed ETEC that were both non-toxigenic and K88-negative did not exhibit mild diarrhea.[16] Smith and Linggood concluded that the diarrhea observed in some of the piglets given $K88^+$ Ent^- forms of a porcine ETEC was the direct result of their presence in high concentration in the small intestines of those piglets and further noted that the incidence and severity of the diarrhea in the piglets given the $K88^+$ Ent^- forms were considerably less than in those given the $K88^+$ Ent^+ forms and would not cause concern among pig farmers.

The composite of positive results observed in the safety/immunogenicity trial with E1392-75-2A prototype live oral vaccine, including strong immunogenicity with a single dose containing ≥10^{10} CFU generated keen interest to undertake a small but pivotal challenge study. A new cohort of 12 adult community volunteers was given a single oral dose containing 10^{10} CFU of prototype vaccine strain E1392/75-2A in preparation for a challenge study with virulent ETEC to assess vaccine efficacy.[74] Ten recipients of the vaccine from whom paired pre-vaccination and post-vaccination samples of intestinal fluid were obtained exhibited significant rises in titer of antibody to CFA/II antigen. The pre-vaccination geometric mean titer (GMT) was 5, while the post-vaccination GMT was 416. Theretofore, in studies at CVD with CF-based vaccine candidates, the direct administration of CFA/II into the proximal jejunum had elicited the most consistent and highest intestinal IgA anti-CFA/II responses. The intestinal SIgA anti-CFA/II titers elicited by the live vaccine were much higher than those observed in persons who received three spaced enteral doses of purified fimbriae.[74] The new cohort of 12 E1392-75-2A vaccinees was challenged one month later, along with a group of six unimmunized control volunteers, with enterotoxigenic strain E24377A, which is of a different O:H serotype (O139:H28) from E1392-75-2A (O6:H16) but expresses CS1 and CS3 fimbriae as well as LT and ST. All six controls developed diarrhea (100% attack rate) but only three of 12 vaccinees (25% attack rate) experienced diarrhea (75% vaccine efficacy;[74,87] $p < 0.005$, 2-tail Fisher's exact test)

Table 7. Experimental challenge of recipients of a single dose of attenuated non-toxigenic *Escherichia coli* prototype vaccine strain E1392–75-2A (serotype O6:H16, CS1,CS3) or control volunteers with enterotoxigenic *E. coli* strain E24377A (serotype O139: H28 expressing LT, ST, CS1 and CS3)[a]

Study group	Diarrheal illness attack rate (%)	Vaccine efficacy	Positive duodenal fluid cultures	Geometric Mean Number of challenge organisms per ml of duodenal fluid[b]
Controls	6/6[c] (100%)	–	6/6	7 x 10^3 [d]
Vaccinees	3/12[e] (25%)	75%	1/12	1x10^1 [f]

[a]Data are derived from Levine et al[29,74,87].
[b]colony forming units (CFU) per ml of duodenal fluid.
[c]vs [e], p = 0.009.
[d]vs [f], p = 0.0004.

(Table 7); furthermore, the diarrheal illness in the three vaccinees was milder than that observed in the controls.

Intensive clinical bacteriology studies involving intubations to collect intestinal fluid specimens from the proximal small intestine showed that the protection was mediated by preventing colonization of this anatomic site. Duodenal cultures were positive in five of six controls (mean, 7,000 organisms/ml) versus only one of 12 vaccinees (10 organisms/ml; $p < 0.004$).[29,87]

Milestone and landmark on the roadmap

These early CVD studies with prototype live oral vaccine strain E1392-75-2A were pivotal in influencing the CVD strategy to develop a live oral vaccine to prevent ETEC diarrhea. The immunogenicity of a single dose of the prototype live vaccine containing $\geq 10^{10}$ CFU was markedly greater than previous forms of delivering CF-based candidate vaccines, particularly in the stimulation of SIgA anti-CF antibodies. Moreover, significant protection was achieved with a single oral dose against an inoculum that elicited a 100% attack rate in control volunteers. And most importantly, intensive clinical bacteriology studies demonstrated that the mechanism of protection involved preventing virulent ETEC from attaching and colonizing the proximal small intestine, the key anatomic site of host-pathogen interaction in the pathogenesis of ETEC diarrheal disease.[88] Qualitiative stool culture positivity was comparable in both protected vaccinees and in controls.

PTL002, PTL003 AND ACE527 (collaboration between United Kingdom and Johns Hopkins University investigators)

Stimulated by the pioneering volunteer studies with E1392-75-2A, Darsley and coworkers undertook to engineer a multivalent ETEC vaccine based on a combination of no more than three attenuated *E. coli* strains that would collectively express CFA/I, CS1, CS2, CS3, CS5, CS6 and the B subunit of LT (LTB). Their ultimate combination vaccine, called ACE 527, contains three highly engineered ETEC strains designated ACAM2025, ACAM2022 and ACAM2027.[89,90] ACE 527 was evaluated in several clinical trials to assess its safety and immunogenicity and in several challenge studies to evaluate its efficacy in preventing ETEC diarrhea.

Prior to undertaking the extensive engineering of strains required to create ACE527, Turner, Darsley and coworkers modified E1392-75-2A as a prototype live oral ETEC vaccine to determine if they could introduce putatively attenuating mutations to diminish the occasional diarrheal adverse reactions that occurred with E1392-75-2A without diminishing the prototype vaccine's strong immunogenicity.[91] To accomplish that goal, they introduced into E1392-75-2A mutations that had resulted in attenuation of virulent *Salmonella* Typhi and *S.* Typhimurium serovars in humans or animals. These mutations included deletions in *aroC* (encoding an enzyme in the aromatic amino acid biosynthesis pathway) that attenuates *S.* Typhi and *S.* Typhimurium for humans,[92–95] and *ompC* and *ompF* (that encode porins) or *ompR* (that regulates expression of *ompC* and *ompF*). These *omp* mutations attenuated the virulence of *S.* Typhimurium in animals.[96,97]

Two derivatives of E1392-75-2A, strain PTL002, which harbors deletions in *aroC* and *ompR*, and PTL003, which harbors deletions in *aroC*, *ompC* and *ompF*, were evaluated for safety, reactogenicity and immunogenicity in a Phase 1 clinical trial in healthy adult volunteers performed at the Johns Hopkins Hospital General Clinical Research Center.[91] Twenty healthy adult volunteers ingested a dose of either 10^7, 10^8 or 10^9 CFU of PTL002 or PTL003. At the 10^9 CFU dose, mild diarrhea occurred in one of five recipients of PTL002 (20%) and in one of six who ingested strain PTL003 (16.7%). All volunteers who received the highest dose of either vaccine mounted IgA anti-CFA/II antibody lymphocyte supernatant responses.

A second clinical trial of PTL002 and PTL003 was then performed at the Vaccine Testing Unit of the Johns Hopkins University School of Public Health.[98] Both vaccines were again generally well tolerated when 40 healthy adults were given a single dose or two doses containing 10^9 CFU, although mild diarrhea occurred following 2 of 31 doses of PTL003. PTL003 also resulted in more sustained colonization and more robust IgA anti-CS1 and anti-CS3 responses than PTL002.[98,99]

To assess the efficacy of attenuated ETEC vaccine strain PTL003, Mckenzie et al randomized 39 healthy adults to receive two 2 × 10^9 CFU doses of freshly grown PTL-003 vaccine in 200 ml of CeraVacxTM buffer or placebo (CeraVacx buffer) on days 0 and 10.[100] The subjects were fasted for 90 min before and after dosing. On Day 28, after a 90 minute fast, 33 subjects imbibed 120 ml of $NaHCO_3$ buffer followed one minute later by 30 ml. of $NaHCO_3$ buffer containing 3 × 10^9 CFU of the challenge strain *E. coli* E24377A (O139:H28, LT/ST, CS1,CS3). Subjects then fasted for an additional 90 minutes. Close monitoring for diarrhea among the challenged volunteers continued over the next 120 hours. ETEC diarrhea occurred among 13 of 16 control subjects (attack rate 81%) versus 3 of 17 vaccinees (attack rate 76%), thereby showing only 6% vaccine efficacy.[100]

Table 8. Experimental challenge of recipients of two spaced doses (21 days apart) of ACE527[a] live oral vaccine, or placebo, with enterotoxigenic *E. coli* strain H10407 (serotype O78:H11 expressing CFA/I, LT and ST) to assess vaccine efficacy[b].

	Vaccinees	Controls	
	Attack rate (%)	Attack rate (%)	Percent vaccine efficacy (95% CI)
Moderate or severe diarrhea[c]	15/29[d](51.7%)	19/27[e] (70.4%)	27% (−13, 52)
Severe diarrhea	14/29[f] (48.3%)	16/27[g] (59.3%)	19% (−33, 50)
Diarrhea of any severity	16/29[h] (55.2%)	20/27[i] (74.1%)	26% (−11, 50)

[a]Strain, ACAM2025, one of three strains constituting the ACE527 vaccine, expresses CFA/I.
[b]These data are summarized from Darsley et al.[90].
[c]Severe diarrhea is defined as at least six grade 3–5 stools or more than 800 grams of loose (grade 3–5) stools within 24 hours.
Moderate diarrhea is defined as four or five grade 3–5 stools or 401 to 800 grams of loose (grade 3–5) stools within 24 hours.
Mild diarrhea is defined as one loose (grade 3–5) stool of ≥ 300 grams or at least two loose stools (grade 3–5) totaling ≥ 200 grams within 24 hours.
[d]vs [e], p = 0.12 (single tail Fisher's exact test); p = 0.18 (two-tail Fisher's exact test).
[f]vs [g], p = 0.29 (single tail Fisher's exact test); p = 0.44 (two-tail Fisher's exact test).
[h]vs [i], p = 0.12 (single tail Fisher's exact test); p = 0.17 (two-tail Fisher's exact test).

ACE527 challenges

Sixty healthy adult volunteers at the Johns Hopkins School of Public Health were randomly allocated to receive a 10^{11} CFU dose of ACE527 live oral ETEC vaccine (N = 36) or placebo (N = 34). Two doses were given 21 days apart. Six of the 27 ACE527 vaccinees (17.6%) developed diarrhea (4 mild, 1 moderate and 1 severe) versus 1 of 27 placebo recipients (14.8%) who had moderate diarrhea.

Overall, 28 of 29 vaccinees (97%) excreted the vaccine strain, including 86% after the first dose and 71% following the second dose. Using a rather modest definition of seroconversion (2.5 rise in titer over baseline), 36% and 47% of vaccinees who received two doses of ACE 527 exhibited seroconversion of serum IgG and IgA anti-CFA/I antibody, respectively and 56% had ≥ 4-fold rises in IgA ALS.

Twenty-eight days following the second dose of ACE527, 29 vaccinees and 27 controls were challenged with 10^7 CFU of virulent ETEC strain H10407 (O78:H11, CFA/I, LT/ST).[90] Following challenge with 10^7 CFU of virulent ETEC strain H10407, the Johns Hopkins investigators found that moderate or severe diarrhea (their primary endpoint) was observed in 19 of 27 placebo recipients (70.4%) and 15 of 29 ACE527 vaccinees (51.7%), yielding a point estimate of 26.6% vaccine efficacy (Table 8). Severe diarrhea was defined as at least six or more loose stools within 24 hrs or >800 mL of loose stool within 24 hrs; moderate diarrhea was defined as four or five loose stools within 24 hr or between 401–800 mL of loose stool over 24 hr. There was also no significant difference in the percent of subjects with severe diarrhea or the percent of subjects with diarrhea of any severity. Notably, the lack of observed efficacy was despite the use of single-tail Fisher's Exact tests in the statistical analyses of the study results. In this review, other investigators who performed controlled challenge studies to assess the efficacy of candidate ETEC vaccines or of passively administered bovine anti-ETEC antibodies typically used two-tail tests in analyzing attack rates between the intervention and control groups post-challenge. Most regulatory agencies request the use of two-tail tests as there exist rare examples when a vaccine unexpectedly showed a significantly increased incidence of severe disease in vaccinees versus control subjects, as occurred with an inactivated RSV vaccine in the 1960s.[101-103]

Milestone and landmark on the roadmap

Although the three-strain ACE527 is a very different vaccine from the E1392-75-2A spontaneous mutant fimbriated non-enterotoxigenic *E. coli* strain that generated much enthusiasm as a prototype live oral vaccine, the modest, non-significant, efficacy results with ACE527 were disappointing. The evolution from E1392-75-2A to ACE527 came in two large steps. The first was the introduction into E1392-75-2A of several mutations in an attempt to diminish diarrheal reactogenicity, resulting in strains PTL002 and PTL003. Deletions in *aroC*, *ompC* and *ompF* were chosen to be the putative attenuating mutations used in subsequent constructs. The second step involved *de novo* engineering of three totally distinct parent strains to introduce the putative attenuating mutations in *aroC, ompC* and *ompF*, while achieving the expression of CFA/I, CS1, CS2, CS3, CS5 and CS6 as a composite among the three vaccine strains. With respect to diarrheal reactogenicity, although PTL003, derived from E1392-75-2A, was tested in clinical trials for safety and immunogenicity, it is not possible to conclude that the introduced mutations attributed to lesser reactogenicity or even if there was less reactogenicity. The doses of PTL003 administered to volunteers contained $5x10^7$, 5×10^8 or 5×10^9 CFU. By comparison, in the early Phase 1 studies with E1392-75-2A the volunteers were given $1x10^9$, $1x10^{10}$, or 6×10^{10} CFU and diarrheal adverse reactions were observed only in the subjects who received ≥ 10^{10} CFU (2 of 15, 13%). Since the trials with PTL003 did not include a randomized group that received the parent E1392-75-2A strain, one cannot conclude that the *aroC, ompC* and *ompF* mutations were exhibiting an attenuating influence. Moreover, these mutations were selected based on their proven ability to attenuate *Salmonella* for humans and *ompC* and *ompF* for mice. The deletion in *aroC* results in an interruption of the aromatic biosynthesis pathway which renders *Salmonella* auxotrophic for 2,3 dihydroxybenzoate when it is intracellular. However, for a non-invasive intestinal pathogen such as ETEC that resides extracellularly within the lumen of the intestine, 2,3 dihydroxybenzoate may be available through dietary intake of raw vegetables. Immunogenicity also correlated with the dose of E1392-75-2A ingested and was maximal when a dose ≥ 10^{10} CFU was ingested.

Although a challenge was performed with PTL003 to assess the efficacy of that vaccine strain and showed no efficacy, the

clinical development program did not include an intermediate challenge study to bridge the reactogenicity and efficacy of E1392-75-2A compared to PTL003 or ACE 527. Such a study might have involved a group of volunteers immunized with a large dose (or two doses) of E1392-75-2A, along with groups that received either PTL003 or ACE527, followed by challenge of the groups of vaccinees and placebo recipients with E23477A. Such a challenge study would have allowed Darsley et al to create a critical bridge to the early challenge studies that showed significant efficacy with ETEC prototype live oral vaccine strain E1392-75-2A. Instead, Darsley et al not only changed the vaccine strain but they selected a different ETEC challenge strain, modified the mode of challenge and multiple variables. This makes it difficult to draw conclusions. Despite the disappointing results reported by Darsley et al with the ACE527 ETEC live oral vaccine, CVD researchers retained confidence in the potential advantages of a live vaccine approach to prevent ETEC disease.

Vaccines based on the expression of ETEC CF and LTB in heterologous bacterial vectors

The fact that prototype ETEC vaccine strain E1392-752A could be highly immunogenic with a single large oral dose, but at a cost of diarrheal adverse reactions (albeit mostly mild) in ~15% of recipients, led investigators at CVD and elsewhere to explore the expression of ETEC CFs in heterologous live vectors including *Salmonella* and *Shigella*. Early studies at CVD explored the expression in attenuated strains of *S.* Typhimurium and *S.* Typhi, since the Typhi live oral vaccine strains had already been shown to be clinically well tolerated and immunogenic in Phase 1 and 2 clinical trials.[104,105]

The attention of CVD investigators, however, soon turned to expression of ETEC CFs in attenuated *Shigella* strains, as the ability to rationally attenuate *Shigella* serotypes was achieving considerable success.[106-113] This provided a broad strategy to accomplish with a single multivalent live vector combination vaccine protection against the major serotypes of *Shigella* that cause disease globally and protection against the majority of ETEC strains incriminated as pathogens. However, this required careful attention to selecting the specific *Shigella* serotypes to be included in the multivalent combination *Shigella*/ETEC vaccine as live vectors as well as a decision on the specific CF antigens to be expressed.

Shigella serotypes included in a combination *Shigella*/ETEC vaccine

In order to keep the number of strains in the combination live vector vaccine to a minimum to minimize the cost of goods and avoid interference among the strains, and after careful review of the *Shigella* serotypes from shigellosis cases isolated during GEMS, we selected five *Shigella* serotypes to serve as live vectors: *S. sonnei, S. flexneri* 1b, *S. flexneri* 2a, *S. flexneri* 3a and *S. flexneri* 6. Collectively, these five strains accounted for 23.7%, 7.5%, 20.2%, 11.0% and 9.4%, respectively, of the 1130 *Shigella* isolates from cases of moderate-to-severe diarrhea.[114] Taking into account only the possibility of direct protection, collectively these five serotypes would theoretically be able to provide protection against 71.8% of the most common *Shigella* pathogens worldwide associated with moderate-to-severe diarrhea. However, the *S. flexneri* 1b, *S. flexneri* 2a and *S. flexneri* 3a vaccine strains share group antigens with all the other *S. flexneri* serotypes (except *S. flexneri* 6 and *S. flexneri* 7a).[114,115] Cross protection from shared group antigens would allow coverage against 990 of the total 1130 (88%) GEMS case-associated *Shigella* isolates.[114] Challenge studies in the guinea pig model and serological studies indicate that cross protection mediated by immune responses to shared group antigens is feasible.[116]

ETEC CFs to be included in a combination *Shigella*/ETEC vaccine

The total number of CF antigens to be expressed collectively in the five *Shigella* live vector strains includes all seven major CFs (CFA/I and CS1, CS2, C3, CS4, CS5, CS6) and one minor CF (CS14).[59] The composition of the CVD multivalent combination *Shigella*/ETEC live vector vaccine is summarized in Figure 1. A pilot lot prepared under Good Manufacturing Processes of the *S. flexneri* 2a component expressing CFA/I and LTB will shortly be evaluated for safety, reactogenicity,

CVD multivalent live vector *Shigella*/ETEC vaccine

Attenuated *Shigella* vaccine strain serotype	ETEC antigens expressed from *Shigella* chromosome
S. sonnei	CS2, CS3
S. flexneri 2a	CFA/I, LTh A2B pentamer
S. flexneri 3a	CS1, CS5
S. flexneri 6	CS4, CS6
S. flexneri 1b	CS14

Figure 1. The composition of the multivalent *Shigella*/enterotoxigenic *Escherichia coli* live oral combination vaccine is summarized in Figure 1. The epidemiologic and immunologic rationale for inclusion of the specific five attenuated *Shigella* live vector serotypes is based on data from Livio et al.[114] The rationale for the specific ETEC colonization factor and toxin antigens expressed by the *Shigella* live vector strains derives from the data of Vidal et al.[59]

immunogenicity and efficacy in preventing ETEC diarrhea due to challenge strain H10407 (that expresses CFA/I, LT and ST) and in preventing shigellosis caused by virulent *S. flexneri* 2a strain 2457T. Positive results will pave the way for testing the full multivalent *Shigella*/ETEC combination vaccine.

Milestone and landmark on the roadmap

A series of volunteer challenge studies performed over many years has paved the way for the definitive testing of one component of the CVD *Shigella*/ETEC live vector multivalent combination vaccine. Should the *Shigella*/ETEC live vector multivalent combination vaccine strategy prove to be well-tolerated, immunogenic and protective in healthy U.S. adults, the vaccine will be evaluated in age de-escalation studies in field sites in several developing countries, ultimately reaching the infant age group. It is anticipated that a three-dose immunization schedule will be required to immunize infants in developing countries. Three-dose schedules of vaccine administered at 6, 10 and 14 weeks or at 10 weeks, 14 weeks and 9 months are being planned.

Protection achieved by passively administered oral antibodies

Strong protection was afforded to newborn piglets against colibacillosis and calves against neonatal diarrhea if the infant animals suckled on sows or cows that had been immunized in late pregnancy with fimbrial antigens.[41-43,117] These pioneering veterinary studies allowed investigators to ask the question of whether protection against ETEC in humans could be achieved if sufficient amounts of specific functional anti-CF, anti-O and anti-LT antibody could reach the mucosa of the proximal small intestine by passive administration, thereby bypassing the vagaries of antigen delivery systems and active immunization regimens. Tacket et al performed at CVD a groundbreaking clinical trial in conjunction with Swiss investigators from Nestlé.[118] An ETEC immunoglobulin concentrate was prepared from the colostrum and milk of dairy cows hyper-immunized with an array ETEC strains of different serotypes expressing the major CFs, LT and ST. During the last eight weeks of gestation, milking cows were injected with 7 or 8 subcutaneous injections of 2×10^9 CFU of the mix of ETEC strains that were heat- or glutaraldehyde-treated, along with Freund's incomplete adjuvant, LT and cholera toxin (CT). The batches were mixed to derive a multivalent product that was assured to have antibodies against various O serogroups, LT, CT, CFA/I and CS3. Milk was obtained from the cows during the first 10 days of lactation post-calving. Milk fat was removed, the remaining skim milk was pasteurized, casein, lactose and salts were removed and the remaining whey-protein solution was concentrated, sterilized and lyophilized to create the final product, a powder that was 85% protein (almost half of which was immunoglobulin). The immunoglobulin powder was mixed with magnesium and ammonium hydroxide to be able to neutralize gastric acid. Single-dose packets were prepared with each containing 3 g of immunoglobulin and 350 mg of antacid salts. The milk immunoglobulin concentrate was shown to have anti-CFA/I at a titer of 1:64,000. A similarly prepared rotavirus immunoglobulin concentrate served as the control preparation.

Twenty healthy adult Maryland community volunteers were randomly allocated to receive immunoglobulin concentrate or placebo (rotavirus immunoglobulin concentrate). Each participating volunteer ingested the anti-ETEC or anti-rotavirus Ig plus buffer preparation three times each day, 15 minutes after each meal for seven days. Two hours after the seventh dose (ingested on the third day of the passive immunoprophylaxis protocol), all 20 volunteers were challenged (after neutralizing stomach acid with $NaHCO_3$ buffer) with 10^9 CFU of virulent ETEC strain H10407 (O78:H11, CFA/I, LT/ST). As summarized in Table 9, nine of the 10 volunteers who received anti-rotavirus Ig concentrate developed diarrheal illness (90% attack rate) versus none of the 10 recipients of the anti-ETEC Ig concentrate (0% attack rate), demonstrating 100% protective efficacy ($p < 0.0001$).

The results of Tacket et al were corroborated years later by Savarino et al who also provided insights on the specificity of the targets of the protective antibodies.[119] Savarino et al assessed the efficacy of anti-adhesin bovine colostral IgG (bcIgG) antibodies against ETEC challenge in volunteers.[119] Healthy adults were randomly allocated (1:1:1) to ingest oral hyperimmune bIgG containing antibodies against the CfaE minor tip adhesin subunit of CFA/I or against purified whole CFA/I fimbriae, or they ingested placebo. Two days before challenge, volunteers began ingesting three times daily one of the investigational products administered in $NaHCO_3$ 15 minutes after each meal; ingestion of products continued for seven days. On the third day, subjects in the three groups were challenged with 1×10^9 CFU of ETEC strain H10407 (O78:H11, LT/ST, CFA/I) with buffer and the occurrence of diarrhea was recorded over 120 hours of follow-up post-challenge.

Diarrhea was observed in 9 of 11 recipients of placebo (82% attack rate), versus 1 of 10 volunteers who ingested anti-CFA/I bcIgG (10% attack rate, protective efficacy = 88%

Table 9. Experimental challenge with 10^9 colony forming units of virulent enterotoxigenic *Escherichia coli* strain H10407 of volunteers who were passively ingesting a bovine immunogloboulin concentrate containing high titers of antibodies against ETEC antigens or a control immunoglobulin concentrate containing anti-rotavirus antibodies.

Passive antibodies ingested by the study group	Diarrheal illness attack rate (%)	Protective efficacy	Subjects with stool cultures positive for ETEC	Geometric Mean number of challenge organisms per gram of stool[a]
Anti-ETEC Ig concentrate	0/10[b] (0%)	–	10/10	1.1×10^8 [c]
Anti rotavirus ETEC Ig concentrate	9/10[d] (90%)	100%	10/10	9.8×10^8 [e]

Data in this table derive from Tacket et al[118].
[a]colony forming units (CFU) per ml of duodenal fluid.
[b]vs [d], $p = 0.009$.
[c]vs [e], $p = 0.0004$.

Table 10. Summary of a challenge with ~10^9 of virulent ETEC strain H10407 (O78:H11, CFA/I, LT/ST) of groups of students volunteers who received passive prophylaxis for seven days with different dosages bovine immunoglobulin concentrate administered with or without $NaHCO_3$ buffer or of placebo. Challenge occurred on day three of the passive prophylaxis regimen.[b.]

	Placebo group	Bovine anti-ETEC Ig, 400 mg tablets plus $NaHCO_3$	Bovine anti-ETEC Ig, 400 mg tablets without buffer	Bovine anti-ETEC Ig, 200 mg tablets without buffer
No. of subjects	14	14	15	14
Attack rate for diarrhea (%)[c]	12/14 (85.7%)	2/14 (14.3%)	3/15 (20.0%)	5/14 (35.7%)
Mean number of diarrheal stools per volunteer with diarrhea (range)	5 (3–10)	3.5 (3–4)	4.7 (2–7)	5 (2-7)
ETEC H10407 isolated from stool post-challenge	12/14 (85.7%)	14/14 (100%)	12/15 (80.0%)	14/14 (100%)

[a]Data in this summary were adapted from Otto et al[120].
[b]Volunteers ingested the ETEC challenge inoculum suspended in 20 ml of $NaHCO_3$ solution immediately after lunch on day 3.
[c]Diarrhea was defined as ≥ 2 loose stools during a 48-hour period within 72 hours of ingesting the challenge inoculm.

[p = 0.002]) and versus 3 of 10 volunteers who ingested anti-CfaE bcIgG (30% attack rate, protective efficacy = 63% [p = 0.03]).[119] This study provided additional evidence of the ability of passively administered anti-CF antibodies to prevent ETEC diarrhea. The trial of Savarino et al and constituted the first clinical trial evidence of the protective efficacy of antibodies directed against a fimbrial tip adhesin.[119]

Milestone and landmark on the roadmap

The study by Tacket et al demonstrated unequivocally that high level protection could be achieved against a potent ETEC challenge if appropriate antibodies in sufficient quantity could be present along the intestinal mucosa. In this instance the antibodies were provided passively.[118] The dilemma faced by vaccine developers is to devise an effective strategy to elicit the appropriate type and quantity of antibodies following active immunization. In the meantime, the study of Tacket et al launched product development for passive protection, one of which, Travelan, is an available commercial product to prevent ETEC travelers' diarrhea (*vide infra*)

Travelan

Tablets consisting of 200 g of lyophilized bovine colostrum powder (BCP) from the colostrum of Australian dairy cows that had been immunized with antigens derived from 14 ETEC strains belonging to O-serogroups associated with travelers' diarrhea and that collectively expressed the six major CFs (CFA/I, CS1, CS2, CS3, CS4, CS5 and CS6) and several minor CFs (CS7, CS12, CS14 and CS17).[120] In total, 90 healthy adult volunteers participated in two randomized, double-blind, placebo-controlled trials to investigate the ability of different tablet formulations to protect against diarrhea following an oral challenge with an O78 ETEC strain. An initial clinical trial involving 30 medical students and other University of Warsaw, Poland student volunteers assessed whether thrice daily ingestion of tablets containing 400 mg of bovine ETEC Ig concentrate administered with $NaHCO_3$ buffer before meals for seven days would protect against experimental challenge with ~10^9 CFU of virulent ETEC strain H10407. Subjects randomly allocated to the placebo group received tablets containing lactose instead of bovine Ig. The challenge inoculum was administered immediately after lunch on the third day. The attack rate for diarrhea was 11 of 15 subjects in the placebo group (73%) versus only 1 of 15 subjects (7%) in the bovine anti-ETEC Ig group, documenting 90.4% protection against ETEC diarrhea.[120]

In a more ambitious second trial, 57 young adult Warsaw students were randomly allocated to receive placebo tablets with $NaHCO_3$ buffer, 400 mg tablets of anti-ETEC Ig with $NaHCO_3$ buffer, 400 mg tablets of anti-ETEC Ig without $NaHCO_3$ buffer, or 200 mg tablets of anti-ETEC Ig without buffer.[120] The results of this challenge study with ~10^9 CFU of ETEC strain H10407 are summarized in Table 10. Significant protection against diarrhea was observed in each group including those who ingested 200 mg tablets of bovine anti-ETEC Ig concentrate thrice daily without buffer. Accordingly, to lower the cost of goods and maximize supply, that dose and presentation (200 mg in caplets) and that regimen became the basis of the commercial product, Travelan. This passive prophylaxis product against ETEC travelers' diarrhea is an approved over-the-counter medicine in Australia and is considered a Natural Health Product in Canada. In the U.S.A. Travelan has self-approved GRAS (generally regarded as safe) status.

Modifications of the ETEC challenge model

From the earliest days of volunteer challenges with ETEC, attempts were made to standardize clinical definitions, methods and procedures so that the experimental challenge models would minimize the number of exposed volunteers required to obtain a clear measure of efficacy, while maximizing repeatability, achieving adequate power to detect a significant difference and minimizing risks to the volunteers. Use of the CVD five-grade classification to document the consistency and character of stools passed by volunteers became widely accepted by other groups of investigators doing challenges. Measurement of the weight of loose stools, with 1 gram approximating 1 ml, also became routine. The definitions of mild, moderate and severe ETEC diarrhea used in some ETEC challenges over the years became identical or very similar among all practitioners of ETEC challenge studies. A small number of challenge strains have been employed preferentially by different research groups for challenges, with LT/ST strains H10407, B7A and E24377A being used most often. More recently, lots of ETEC challenge strains prepared under Good Manufacturing Practices

(GMP) have become available to increase further the standardization across time and to offer the possibility of future multi-site challenge studies by use of an identical stored inoculum. This follows the strategy crafted for multi-site cholera challenges that has accelerated licensure of a live cholera vaccine by the U.S. Food and Drug Administration.[121,122] The administration of $NaHCO_3$ (or a similar buffer) pretreatment to neutralize gastric acid, thereby allowing lower inocula to be administered to volunteers and increasing the consistency of clinical responses, has become routine even among the most recent research groups that perform ETEC challenge studies.[30,47,53,70,118,120,123] Attempts to increase further the consistency of experimental challenge by lengthening the period of fasting prior to ingestion of ETEC inocula and evaluating buffers other than $NaHCO_3$ have also been pursued.[37]

Concluding statement

Since the 1970s, experimental challenge studies with virulent wild type ETEC have allowed insights into pathogenesis, human immune response to putative ETEC virulence determinants, identification of human biological risk factors and the means to assess preliminarily the efficacy of candidate ETEC vaccines. Stocks of various virulent challenge strains prepared under Good Manufacturing Practices are stored and available as reagents to facilitate clinical evaluation of ETEC vaccines. Selective use of experimental challenge studies can accelerate the clinical development of ETEC vaccines and contribute to the evidence base on safety, immunogenicity and efficacy to be reviewed by national regulatory agencies on the path towards licensure.

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