Colonization Factors in Enterotoxigenic *Escherichia coli* Strains in Travelers to Mexico, Guatemala, and India Compared with Children in Houston, Texas

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Abstract. Enterotoxigenic *Escherichia coli* (ETEC) can be attributed to around 200 million diarrheal episodes and 380,000 deaths in the developing regions. Travelers' diarrhea occurs in 15–40% of travelers to developing regions with ETEC being the most important etiologic agent. This study aims to describe the distribution of enterotoxins and colonization factor (CF) profiles of ETEC isolates from stool samples of adult travelers acquiring diarrhea in Mexico, Guatemala, and India and a group of children with acute diarrhea in Houston, TX, between 2007 and 2012. The heat-labile/heat-stable (LT/ST) enterotoxins and CFs from 252 patients were determined using polymerase chain reaction assay. Among the 252 ETEC isolates, 15% were LT-only, 58% were ST-only, and 28% produced both LT and ST. The distribution of LT-only (12–15%) and ST-only (55–56%) isolates was similar between Latin American and Indian sites. The most prevalent CF was CS21, expressed in 65% of the isolates followed by CS6 (25%) and CS3 (17%). Among the international travelers, 64% of the ETEC isolates expressed CS21. CS21 was expressed in 46% of isolates from Latin America compared with 96% of isolates from India ($P < 0.0001$). CS21 was expressed in 85% isolates from Houston children. CS21 was increasingly found in ST-only ($P = 0.003$) and ST/LT ($P = 0.026$) ETEC compared with LT-only ETEC. High frequency of finding CS21 among recent isolates of ETEC over a wide geographic distribution warrants additional studies on this CF. Highly conserved CS21 is an important target for potential multivalent ETEC vaccines.

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is the most common bacterial enteropathogen that causes diarrhea in both children < 5 years of age and among adults from developing countries visiting in these regions ("travelers' diarrhea").¹ Among children < 5 years of age in low-income countries, ETEC and rotavirus are the most common causes of diarrhea.² An estimated 200 million diarrheal episodes and 380,000 deaths are attributed to ETEC in the developing world.^{3,4} Travelers' diarrhea is the most common illness reported among people from industrialized regions visiting developing countries occurring in 15–40% depending on region ETEC being the most important etiologic agent.⁵

ETEC virulence factors (VFs) include enterotoxins, both heat-stable (ST) and heat-labile (LT) toxins and colonization factor (CF).⁶ The CFs are either fimbrial, fibrial, or non-fimbrial in structure that facilitate the adhesion of the bacteria to the intestinal cells, to colonize and to cause diarrhea. To date, over 25 CFs have been identified, but only few are considered more prevalent than others.⁶ Antibodies against CFs are thought to be protective against ETEC infection and thus determining the most prevalent CFs in developing countries may help in developing vaccine candidates that are more effective for children and travelers to these regions.^{7–10} This is particularly important for ST-producing ETEC strains as ST is only poorly antigenic and successful immunoprophylaxis depends on the presence of CFs on infecting ETEC strains.¹¹ Up to 51% of ETEC isolates from cases of travelers' diarrhea elaborate ST-only.¹² A challenge to vaccine development is the temporal, regional, and population-specific variabilities in ETEC CFs expressed.¹³

There is heterogeneity in the ETEC strains circulating among the pediatric population in developing countries and the strains infecting traveler's to that country as supported in a recent study in Guatemala that toxins and CF do not completely overlap among indigenous children and adult travelers,¹⁴ and is further supported by the systematic review performed by Isidean and others.⁶ Thus, a vaccine for ETEC needs to have broad coverage for both travelers to, and pediatric populations in the developing world. The distribution of enterotoxins and CFs from recently identified ETEC strains from diverse regions of the world should be characterized as we develop effective multivalent vaccines to address all prevalent pathotypes in target populations of interest.

The objective of this study was to describe the distribution of enterotoxins and CF profiles of ETEC isolates collected from stool samples of adult travelers acquiring diarrhea in Mexico, Guatemala, and Goa, India. Although not directly comparable, we included a group of children with acute diarrhea in Houston, TX. All studies took place between 2007 and 2012.

METHODS

Laboratory methods. We collected stool samples from adult travelers to Mexico, Guatemala, and India participating in a series of clinical trials carried between 2007 and 2012. To expand our studies of geographic diversity, we also include in the study inpatient children with acute watery diarrhea seen at Texas Children Hospital in Houston, TX. Stools from all subjects were cultured on MacConkey agar with identification of ETEC, ST, LT, and ST/LT-producing strains by published methods.¹⁵ For this study, isolates identified as ETEC in the earlier studies were recultured on MacConkey agar and isolated for biochemical testing with the analytical profile index 20 strips (bioMérieux, Cambridge, MA).¹⁶ We determined a seven-digit identifier on the basis of 21 biochemical reactions to confirm the organism as *E. coli*.

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To reconfirm the LT/ST toxin type and to determine the CF type of the ETEC isolates, we used multiplex polymerase chain reaction (PCR) to detect structural genes for LT, ST, and 10 CFs using the protocol developed by Rodas and others.¹⁷ The PCR mixture of 25 µL contained 10–100 ng boiled bacterial DNA mixed with 2 mM MgCl₂, 400 nM deoxynucleoside triphosphate, 8–10 forward and reverse primers at a concentration of 200 nM of each primer, 1 µL Taq polymerase, and 1× PCR buffer. The initial denaturation occurred at 94°C (1 minute), followed by 35 cycles of amplification (94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 1 minute), and finally, 5 minutes at 72°C. We resolved the PCR products on 3% agarose gels and visualized under ultraviolet light.^{17,18}

Statistical analysis. We calculated two-tailed Fisher's exact test for the analysis of independence among different strains expressing LT/ST toxins and CFs. We used STATA version 13 (StataCorps LP, College Station, TX); a *P* value less than 0.05 was considered significant.

RESULTS

We tested ETEC isolates obtained from 252 patients with diarrhea studied between 2007 and 2012 for CFs using multiplex PCR. Among those isolates, 219 (87%) were from adult travelers developing diarrhea in Guatemala, Mexico, or India, whereas 33 (13%) isolates were from children admitted with acute watery diarrhea in the Texas Children Hospitals in Houston. Among the 252 ETEC isolates, 37 (15%) produced LT-only enterotoxin, 145 (58%) ST-only enterotoxin, and 70 (28%) both LT and ST type enterotoxin (Table 1). The distribution of LT-only ETEC strains was similar between the study areas (12–15% of isolates). The proportion of ST-only ETEC was similar when the Latin American sites were compared with India (55% and 56%). Two hundred and two of the 252 ETEC isolates (80%) were shown to express at least one CF. The most prevalent CF produced was CS21, which was expressed in 165 (65%) of the isolates. The other frequently expressed CFs were CS6, which was expressed in 63 (25%) of isolates, and CS3, which was expressed in 44 (17%) of the ETEC strains tested. Among the international travelers with ETEC diarrhea, 137 of 219 (64%) of the ETEC isolates expressed CS21. The ETEC isolates from Latin America expressed CS21 in 67 of 146 (46%) strains compared with 70 of 73 (96%) (*P* < 0.0001) strains isolated from travelers acquiring diarrhea in India. CS21 was expressed in 28 of 33 (85%) isolates collected from Houston children. CS6 occurred more commonly in ETEC diarrhea occurring in Latin America compared with India, 47/146 (32%) versus 14/73 (19%) (*P* = 0.0007). Of the 219 ETEC isolates identified in adults with travelers' diarrhea, 162 (74%) expressed either CS21 or CS6. Of these travelers' diarrhea ETEC strains, 137 (62.5%) expressed only CS21, 61 (28%) expressed only CS6, and 36 (16%) produced both.

Expression of CFs differed by ETEC toxin type (Table 2). Twenty-one (57%) LT-producing isolates, 106 (73%) ST-producing ETEC isolates, and 38 (54%) LT/ST ETEC strains produced CS21 (*P* = 0.0122). In subgroup analyses, CS21 expression was increased in occurrence in ST-only (*P* = 0.003) and ST/LT (*P* = 0.026) ETEC.

TABLE 1

Toxin type and CF profiles of 252 ETEC isolates obtained from individual cases of travelers' diarrhea of adults in developing regions and pediatric cases of diarrhea in Houston, 2007–2012

Location	LT-only	ST-only	LT/ST	CS1	CS2	CS3	CS4	CS5	CS6	CS7	CS17/19	CS21	CFA1
ETE, n (%)	N = 37 (15)	N = 145 (58)	N = 70 (28)	N = 25 (10)	N = 22 (9)	N = 44 (17)	N = 4 (2)	N = 1 (0.3)	N = 63 (25)	N = 0 (0)	N = 1 (0.7)	N = 165 (65)	N = 3 (1)
Guatemala and Mexico, N = 146 (57)	22 (15)	81 (55)	43 (29)	20 (14)	19 (13)	20 (14)	0 (0)	1 (0.7)	47 (32)	0 (0)	1 (0.7)	67 (46)	3 (2)
India, N = 73 (29)	11 (15)	41 (56)	21 (29)	5 (7)	3 (4)	24 (33)	3 (4)	0 (0)	14 (19)	0 (0)	0 (0)	70 (96)	0 (0)
United States, N = 33 (13)	4 (12)	23 (70)	6 (18)	0 (0)	0 (0)	0 (0)	1 (3)	0 (0)	2 (6)	0 (0)	0 (0)	28 (85)	0 (0)
Total ETEC, N = 252	37 (15)	145 (58)	70 (28)	25 (10)	22 (9)	44 (17)	4 (2)	1 (0.3)	63 (25)	0 (0)	1 (0.3)	165 (65)	3 (1)

CF = colonization factor; ETEC = enterotoxigenic *Escherichia coli*.

TABLE 2

Distribution of CFs and toxin types seen in 252 ETEC isolates obtained from individual cases of travelers' diarrhea in adults and in pediatric cases of diarrhea in Houston, 2007–2012 (CFs found in only 1–3 ETEC strains removed)

CF (n)	LT-only	ST-only	ST/LT	P value
	N = 37 (%)	N = 145 (%)	N = 70 (%)	
CS1 (25)	2 (5)	7 (5)	16 (23)	0.0003
CS2 (22)	1 (3)	7 (5)	14 (20)	0.0009
CS3 (44)	6 (16)	9 (6)	29 (41)	0.0000
CS4 (4)	0 (0)	3 (2)	1 (1)	0.0028
CS6 (63)	7 (19)	31 (21)	25 (36)	0.0554
CS21 (165)	21 (57)	106 (73)	38 (54)	0.0122

CF = colonization factor; ETEC = enterotoxigenic *Escherichia coli*.

CS6 expression was common in all ETEC toxin types with a slightly higher frequency seen in ST/LT ETEC strains ($P = 0.0554$). ST/LT ETEC were also more likely to express CS1, CS2, and CS3 compared with LT-only and ST-only toxin types ($P = 0.0003$, 0.0009 , and < 0.0001 , respectively).

Among the 50 ETEC that did not express any CF, 30 (60%) were ST ETEC, 12 (24%) were LT ETEC, and eight (16%) were ST/LT ETEC ($P = 0.0323$).

DISCUSSION

ETEC is the most important cause of travelers' diarrhea in Latin America, Africa, and southern Asia.⁵ In this study, we identified ST as the most frequent toxin type expressed among the studied ETEC isolates from four regions of the world. This is important because ST is poorly antigenic or nonantigenic, shares epitope homology with the human endogenous peptides of the guanylin family, and has yet to be incorporated into a vaccine construct; however, efforts of with ST-toxoid fusions are actively being developed.^{19,20} Our finding that ST as the most common ETEC toxin is in agreement with a systematic review carried out by Isidean

and others⁶ In their study among travelers acquiring diarrhea in Latin America, the proportion of ETEC expressing ST was 67% and for travelers in south Asia it was 56%. In our study, we found that 56% of ETEC identified were ST-producing strains among travelers to Mexico, Guatemala, and India. Our results, specifically for Guatemala and Mexico, are consistent with a recent report that identified ST-only ETEC among travelers visiting Guatemala in the years 1999 to October 2003, and suggests stability in the epidemiology of ETEC in this region.¹⁴ Because ST is poorly immunogenic, vaccine manufacturing efforts to date have focused on the immunogenic LT component for vaccine development.^{21,22} Our finding indicates that LT, either alone or in combination with ST, was expressed in less than half of ETEC isolates. Thus, for effective vaccination coverage using an LT immunogen, multiple CFs or other VFs will be required to provide protection against ST-only ETEC strains.

Among the CFs studied in this study, CS21 was the most frequent CF identified, found in 165 of 252 (65%) of the ETEC isolates in all groups. Among the isolates from international travelers, 63% of the ETEC strains expressed CS21. In a small number of Houston children, 85% of ETEC isolates were CS21 positive. Expression of CS21 has been reported as the most frequent CF in ETEC strains, especially for Latin America/Caribbean and Middle East/north African region.⁶ CS21 has been shown to be important in endemic pediatric diarrhea,²³ identified in half of the strains identified. Limited data are available for CS21 and its role in ETEC virulence. CS21 is a type IV pilus that has major structural subunit called LngA protein; the expression of which induces bacterial self-aggregation, protection from environmental stressors, and cell adhesion.²⁴ In their experimental animal model, Guevara and others confirmed the role of CS21 in the pathogenesis of ETEC diarrhea.²⁵ More recently, the ETEC isolates with CS21 has been linked with self-aggregation, biofilm formation, and adherence to intestinal cells among Bangladeshi children with diarrhea.²⁶

Apart from CS21, the other two CFs that were more frequently expressed were CS6 (25%) and CS3 (17%). CS6 is a structural gene important in binding and pathogenicity of ETEC, which has two structural subunits²⁷ showing differential binding capacity that may be linked with pathogenicity.^{28–30} In a study of international travelers returning to Spain, 30 of 52 (58%) of ETEC strains isolated were CS21 positive and 14 of 52 (27%) were CS6 positive.³¹ A study of a killed oral vaccine combining *Vibrio cholerae*-binding toxin plus ETEC CFs, carried out in Mexico and Guatemala, was of limited value because it did not contain CS6 found in prevalent ETEC strains encountered in the study.²² The lead scientific group working with this vaccine is now adding CS6 to the vaccine.³²

In looking at the ST-only strains in this study, CS21 expression was seen in 106/164 (65%) of ST-ETEC strain while CS6 expression was seen in 31/164 (19%) of the ST-ETEC strains. In our study, 112 (77%) ST-ETEC produced either CS21 or CS6. Adding CS21 and CS6 to any future LT-based vaccine developed is advised.

There are two lead cellular ETEC vaccine approaches under development. The killed whole-cell vaccine EtVax (Scandinavian Biopharma, Solna, Sweden) contains an admixture of ETEC strains expressing CFA/I, CS3, CS5, CS6, and the hybrid protein and the hybrid protein LCTBA.³³

FIGURE 1. Hypothetical vaccine coverage assuming LT-only and LT/ST toxin type with common colonization factors. LT = heat labile; ST = heat stable.

The other vaccine ACE527 (TD Vaccines A/S, Skorpning, Denmark) is an admixture of three live-attenuated *E. coli* expressing CFA/I, CS1, CS2, CS3, CS5, CS6, and LT-B.³⁴ Given these candidate vaccines under development, we estimated the proportion of ETEC disease that would have been covered from these vaccines under the following assumptions (Figure 1). Assuming that an anti-LT immune response from both vaccines would cover LT-only-associated ETEC disease, the EtVax and ACE527 vaccines would cover 46% and 54%, respectively. Expanding the assumption that the LT component would cover both LT and LT/ST strains would only add an additional 6% coverage to each of these vaccine constructs. Although these are estimated coverage levels, vaccine efficacy is unlikely to be 100% against vaccine preventable outcomes, thus to achieve broad coverage against travelers' associated ETEC, additional CFs and/or other virulence determinants are likely needed.

In addition to the commonly expressed CFs, ETEC also express nonclassical VFs. Among the nonclassical VFs are the EAST1 and EatA protein which have been found in 65% and 48% of the isolates among Spanish travelers³⁵. We did not test for any nonclassical VF, which is an important limitation of the study. Determining these additional VFs could prove valuable in developing a broadly protective vaccine.

In conclusion, findings of common expression of CS21 and CS6 among recent isolates of ETEC causing travelers' diarrhea over a wide geographic distribution supports the need to perform additional studies of CFs and ETEC diarrhea. If our findings are supported by other studies, highly conserved CS21 and CS6 should be included in future multivalent ETEC vaccines.

Received May 19, 2016. Accepted for publication August 23, 2016.

Published online November 14, 2016.

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