Enterotoxigenic *Escherichia coli* with STh and STp Genotypes Is Associated with Diarrhea Both in Children in Areas of Endemicity and in Travelers[▽]

Ingrid Bölin,^{1*} Gudrun Wiklund,¹ Firdausi Qadri,² Olga Torres,³ A. Louis Bourgeois,⁴ Stephen Savarino,⁵ and Ann-Mari Svennerholm¹

Department of Microbiology and Immunology, and Göteborg Vaccine Research Institute (GUVAX), Göteborg University, Göteborg, Sweden¹; Laboratory Sciences Division, International Centre for Diarrhoeal Disease Research, Dhaka, Bangladesh²; Laboratorios de Microbiología INCAP, Ciudad de Guatemala, Guatemala³; Department of International Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland⁴; and Enteric Diseases Department, Naval Medical Research Center, Silver Spring, Maryland⁵

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Enterotoxigenic *Escherichia coli* (ETEC) is an important cause of diarrhea among children in developing countries and in travelers to areas of ETEC endemicity. ETEC strains isolated from humans may produce a heat-labile enterotoxin (LT) and two types of the heat-stable enterotoxin STa, called STh and STp, encoded by the *estA* gene. Two commonly used assay methods for the detection of STa, the infant mouse assay or different enzyme-linked immunosorbent assays, are unable to distinguish between the two subtypes of ST. Different genotypic methods, such as DNA probes or PCR assays, may, however, allow such discrimination. Using gene probes, it has recently been reported that ETEC strains producing STp as the only enterotoxin are not associated with diarrhea. In this study, we have used highly specific PCR methods, including newly designed primers for STh together with previously described STp primers, to compare the relative distribution of STh and STp in ETEC isolated from children with diarrhea in three different geographically distinct areas, i.e., Bangladesh, Egypt, and Guatemala, and from travelers to Mexico and Guatemala. It was found that ETEC strains producing STp were as commonly isolated from cases of diarrhea as strains producing STh both in Egypt and Guatemala, whereas STp strains were considerably less common in Bangladesh. No difference was found in the relative distribution of STh and STp in ETEC strains isolated from travelers with diarrhea and from asymptomatic carriers. Irrespective of ST genotype, the disease symptoms were also similar in both children and travelers.

Enterotoxin-producing *Escherichia coli* (ETEC) is an important cause of diarrhea among children in developing countries and in travelers to areas of ETEC endemicity (2, 8). ETEC strains isolated from humans may produce a heat-labile enterotoxin (LT), a heat stable toxin (ST), or both LT and ST (14). ST may be classified into two major genotypes, i.e., STa and STb; typically, ETEC strains isolated from humans produce STa (STI) encoded by the *estA* gene, whereas STb (or STII) is predominantly produced by animal ETEC strains. Two different subtypes of STa exist, i.e., STh (or STIb) and STp (or STIa) (12). These designations were chosen, since STh previously was considered to be mainly of human origin and STp was considered to be of porcine origin. Several allelic forms of the *estA* gene exist, and both alleles encoding STp and STh have been found among human ETEC isolates (9). These two variants of STa are very similar in structure and function. STh consists of 18 amino acids and STp of 19 amino acids; both variants contain 6 cysteine residues and, accordingly, most epitopes in the ST molecule are conformational (12). Both STh and STp bind to specific receptors and the intestinal brush

border membrane of the small intestine to activate the guanylyl cyclase-cGMP system, which results in diarrhea (6).

Two STa detection methods that have been widely used, i.e., the infant mouse assay (3) and different enzyme-linked immunosorbent assays (ELISAs) (4, 20), do not allow discrimination between the two subtypes of STa. Hence, the relative distribution of the STh and STp genotypes among human ETEC isolates has infrequently been reported. However, by means of genotypic assays, such as DNA probes, the two subtypes of STa can readily be distinguished (10, 17). In a recent study from Guinea-Bissau of ETEC in young children that used such gene probes, it was reported that only ETEC strains producing STh alone or in combination with LT, but not STp-producing strains, were associated with diarrhea (18). To test whether this is also the case for ETEC isolated from children in other areas of ETEC endemicity and in ETEC from travelers, we have compared the relative distribution of STh and STp in ETEC isolated from children with diarrhea in three geographically distinct locations, Bangladesh, Egypt, and Guatemala. We also compared the relative distribution of STh and STp in ETEC strains isolated from the stool of patients with diarrhea and in routine specimens from travelers to Guatemala and Mexico. These strain comparisons were done by using PCR detection methods. New primers for STh PCR were designed and evaluated together with a previously described STp PCR primer set (22). Using these PCR methods, we now report that ETEC

* Corresponding author. Mailing address: Dep. Microbiology and Immunology, Sahlgrenska Academy, Göteborg University, P.O. Box 435, SE-405 30 Göteborg, Sweden. Phone: 46 31 7736211. Fax: 46 31 7736210. E-mail: ingrid.bolin@microbio.gu.se.

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No	ETEC strain	Toxin profile	Reference
1	Takeda 105	STh	WHO ^b
2	309900-2	SThLT ^a	(5)
3	259325-1	SThLT ^a	(5)
4	278556-1	SThLT ^a	(5)
5	VM75688	SThLT ^a	(2)
6	E11881/9	STp ^a	(7)
7	Takeda 101	STp	WHO ^b
8	VX68011	STpLT ^a	(2)
9	VM66252	STpLT ^a	(2)
10	VM66257	STpLT ^a	(2)
11	E17018/A	SThLT ^a	(7)

FIG. 1. STh and STp PCR of reference strains. The lane numbers are the same as the strain numbers in the table. Strains listed in the table were used in studies from references 2, 5, and 7. a, toxin profile was determined by gene probe assay by Steinsland et al. (17); all strains tested by the gene probes in Bergen, Norway, had been obtained from A.-M. Svennerholm's laboratory. b, strain was obtained as a reference strain collection from the WHO with ST genotypes indicated; the collection was originally prepared by Y. Takeda (21).

strains producing STp are commonly associated with diarrhea both in Egypt and Guatemala and also that there is no difference in the relative distribution of STh and STp genotypes among ETEC isolated from travelers with diarrhea or from asymptomatic individuals visiting the same areas.

MATERIALS AND METHODS

ETEC strains. Previously characterized ETEC strains that were used as reference strains are listed in Fig. 1. The number of clinical ETEC isolates of different origins, subjected to toxin analyses, is described in Results and in Tables 1 to 3. The strains from Bangladesh were isolated from patients seeking care at the hospital of the International Centre for Diarrheal Disease Research, Center for Health and Population Research in Dhaka, for moderate to severe diarrhea during the period of 2001 to 2003. The ETEC strains that were isolated were initially tested for STa and LT production by means of monoclonal antibody-based GM1-ELISA methods (19, 20) at the International Centre for Diarrheal Disease Research and, subsequently, in Sweden; 52 strains that produced STa alone or together with LT that were isolated during the study were included in the present analyses. The Egyptian ETEC strains represented a systematically selected sample of ETEC strains isolated from children with diarrhea in a recent ETEC vaccine trial in Egypt (13). All strains were initially tested at Naval Medical Research Unit no. 3, Cairo, Egypt, for STa and LT by means of GM1-ELISA methods, and all STa-positive strains were sent to Göteborg University for confirmatory analyses as described below. Every fifth strain that was shown to be STa positive by the phenotypic analyses was included in the study, i.e., 46 STa or STa/LT *E. coli* isolates. Additional enteropathogens were detected in three cases (two *Campylobacter*, one rotavirus).

The ETEC strains from Guatemala were either collected during an ETEC vaccine trial in American travelers going to Guatemala or Mexico for language studies from 2000 to 2001 (A. L. Bourgeois, J. Halpern, B. Gustafsson, A.-M. Svennerholm, O. Torres, J. Belkind-Gerson, and D. A. Sack, 45th Intersci. Conf. Antimicrob. Agents Chemother., abstr. G-408, 2005) or in an ongoing study of children with diarrhea in a village close to Guatemala City, Santa María de Jesús, during 2002 to 2003. The ETEC strains from these studies were analyzed by Laboratorios de Microbiología INCAP, Ciudad de Guatemala, Guatemala, or the Department of International Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, Md., and strains that were positive for STa were included in the study. All STa-positive ETEC isolates that had been isolated from children with diarrhea at the time of the study and a randomized sample of STa-positive ETEC strains, including both ST-only and ST/LT, from travelers with diarrhea as well as from routine specimens collected during these studies were included for analyses of the two STa genotypes.

Clinical data regarding symptom scores, duration of disease, and number of unformed stools in 18 cases of traveler's diarrhea were reported by the subjects during the disease episode. The symptom score is the sum of the maximum intensity ratings for gastrointestinal and general systemic symptoms present at any time during the episode. The following gastrointestinal and general symptoms were scored: abdominal pain/cramps, gas, anorexia, nausea, vomiting, gurgling stomach, urgency, fever, headache, muscle aches, and chills. The following intensity scale was used to score symptoms: 0, not present; 1, mild; 2, moderate; 3, severe. Clinical data were obtained from 28 Guatemalan children regarding duration, number of unformed stools, and whether symptoms were absent or present (nausea, vomits, abdominal pain, fever, and excretion of mucus).

Strains were cultured on horse blood agar plates overnight. After having been shown to be ST positive or ST/LT positive by means of GM1-ELISA methods (19, 20), all ETEC strains were stored at -70°C in LB broth with glycerol. Only strains that were positive for STa in confirmatory analyses shortly before the PCR analyses in Göteborg were included in the studies.

GM1-ELISA methods for detection of STa and LT. Five individual lactose-fermenting *E. coli* colonies from each clinical specimen were inoculated in GM1-coated ELISA plates containing 100 μl /well of LB broth with 45 $\mu\text{g}/\text{ml}$ lincomycin and 2.5 mg/ml glucose (19, 20). Thereafter, the plates were incubated at 37°C with shaking (200 rpm) overnight. The next day, 50 μl of the culture of each colony was transferred to GM1-coated ELISA plates that had been incubated with an ST-CTB conjugate for 1 h (15). After washing, 50 μl of the supernatant from the original GM1-coated ELISA plates was transferred to corresponding wells in the ST-CTB-coated plates. After transfer of a specific anti-STa monoclonal antibody (ST1:3), 50 μl per well, plates were incubated with gentle shaking for 90 min. The original GM1-coated plates used for culture overnight were washed, and a specific anti-LT monoclonal antibody (39:13:1), 100 μl , was added to each well. After incubation of all plates for 90 min, a goat anti-mouse immunoglobulin G-horseradish peroxidase conjugate (The Jackson Laboratory, Göteborgs Termometerfabrik, Göteborg, Sweden) was added to the plates. After further incubation for 90 min, the plates were developed with H_2O_2 -OPD substrate and the plates were read at 450 nm. STa-positive isolates were identified as cultures inhibiting absorbance of $\geq 50\%$ compared to the absorbance of negative reference strains, and LT-positive strains were identified as those causing an absorbance value of ≥ 0.1 above background.

PCR. Total DNA was extracted from ETEC strains by harvesting bacteria from 2 ml overnight broth culture. After centrifugation, the bacterial pellet was resuspended in 100 μl of sterile water and boiled for 10 min. The lysate was centrifuged, and the supernatant (DNA sample) was collected and stored at -20°C until used. In cases where no STp or STh PCR products were obtained with this crude DNA preparation, total DNA was also prepared with the QIAGEN DNeasy tissue kit (VWR International, Stockholm, Sweden) according to the manufacturer's instructions for bacterial samples.

All primers were purchased from Cybergene AB, Stockholm, Sweden. STh forward primers STh1Bf (5'-AGTGGTCCTGAAAGCATG-3'), STh5f (5'-TCA CCTTTCCTCAGGAT-3'), and STh7f (5'-AACCAGTAGAGTCTTCAAAA G-3') and reverse primers STh2r (5'-CAACAAAGCAACAGGTAC-3') and STh6r (5'-TACAAGCAGGATTACAACAC-3') were designed from the STh/ST1b sequence (GenBank accession number M34916). Amplification with STh primers, STp (ST1a) primers STp-F1 and STp-R2 (22), and STI primers JW14 and JW7 (16) was performed in a total volume of 50 μl Taq polymerase buffer (10 mM Tris, pH 8.3, 50 mM KCl, and 1.5 mM MgCl_2) containing 10 pmol of each primer, 0.2 mM concentrations of all four deoxynucleotides (Roche Diagnostics Scandinavia AB, Bromma, Sweden), and 1.25 U of Taq polymerase (Sigma-Aldrich Sweden AB, Stockholm, Sweden). One microliter of DNA sample was used. The amplification steps were 94°C for 5 min; 24 cycles consisting of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min; and four

TABLE 1. STa genotype distribution in ST-only and ST/LT *E. coli* strains isolated from diarrhea cases in different countries and age groups

Country(ies) (patient group) of origin	Number (%) of strains of <i>E. coli</i> type and genotype								
	ST only			ST/LT			Total ST		
	Total	STh	STp	Total	STh	STp	Total	STh	STp
Bangladesh (children)	11	11 (100)	1 ^a (9)	41	39 (95)	3 ^a (7)	52	50 (96)	4 (8)
Egypt (children)	25	17 (68)	8 (32)	21	12 (57)	9 (43)	46	29 (63)	17 (37)
Guatemala (children)	19	12 (63)	7 (37)	9	5 (55)	4 (44)	28	17 (61)	11 (39)
Guatemala and Mexico (adult travelers)	12	2 (17)	10 (83)	18	17 (94)	2 ^a (11)	30	19 (63)	12 (40)

^a One strain positive for both STh and STp.

cycles with increased annealing and elongation times, i.e., 52°C for 2 min and 72°C for 10 min, respectively. The PCR products were analyzed by electrophoresis on 2% agarose gels.

DNA probe hybridization assay. STh and STp genes were analyzed by hybridization with DNA probes produced by PCR amplification of plasmids containing the cloned genes, kindly provided by H. Sommerfelt and H. Steinsland, University of Bergen, Norway. The hybridization was performed as previously described (17).

Statistical analyses. The comparison of disease characteristics of STh and STp infections in travelers was performed with the Prism software system (GraphPad Software, Inc., San Diego, California) using the nonparametric Mann-Whitney test.

RESULTS

STh and STp PCR. Phenotypically STa-positive ETEC strains from Guatemala producing STa only and not LT, as determined by GM1-ELISA, were analyzed for the presence of the STh gene with primers STh5f and STh6r. These analyses revealed that some of these strains were negative for STh (Fig. 2). Lowered annealing temperature (i.e., from 52°C to 48°C) in the amplification steps did not increase the number of STh-positive strains. The negative strains all produced ST but not LT (ST only), as determined by ELISA. Two other forward primers (SThIBf and STh7f) in combination with the reverse primers STh6r and STh2r were then tested on the same set of both STh-positive and -negative strains to investigate if there were sequence deviations in the STh gene of ST-only strains that abolished primer binding. Again, no PCR products were obtained from the previously negative, ST-only strains using these primers (data not shown) but only from the strains positive with the first tested primer pair. The primer combination SThIBf plus STh6r was used in subsequent experiments, since it gave a single band with all of the STh-positive strains.

Earlier reports have found STp-positive human ETEC strains by DNA probe hybridization (11, 18). To test the possibility that the ST-only strains that were negative for STh in the above analyses carried the STp gene instead of STh, PCRs

with STp-specific primers were performed. The result showed that most of the STh-negative strains were positive for STp by PCR (Fig. 2).

The specificity of the STh and STp primer sets was tested by PCR analyses of previously characterized ETEC strains. A clear discrimination between STh-positive and STp-positive strains was observed in agreement with previously determined DNA hybridization results (Fig. 1). Only one strain (E17018/A), previously described as STh positive, was found to contain both genes. Primers JW14 and JW7 (16) were also tested, but no PCR products were detected from any of the strains.

Prevalence of STh and STp in ETEC strains from countries of endemicity. In addition to ETEC strains from Guatemala, the study was extended to include both STa-only and LT/STa strains from children from two other countries of endemicity, Bangladesh and Egypt. All of the strains (in total, $n = 126$) were obtained from children with diarrhea. We also tested 52 strains that had been collected from symptomatic and asymptomatic travelers in Guatemala. In total, 178 strains isolated at different time points from the different locations and from different categories of subjects were analyzed with STh primers SThIBf and STh6r and the STp primers STp-F1 and STp-R2. When the prevalence of STh and STp genotypes among strains from symptomatic children were compared, it was found that as many as 37% and 39%, respectively, of the strains from Egypt and Guatemala were positive for STp, whereas the proportion of STp strains was markedly lower in Bangladesh (Table 1). Thus, only 8% of the ST-positive strains were positive

TABLE 3. Severity of disease caused by STh- and STp-positive ETEC strains in travelers to Mexico and Guatemala and Guatemalan children^a

Study group and ST genotype (<i>n</i>)	Duration (days)	Symptom score or symptoms present ^b	No. of unformed stools
Travelers			
STh (9)	7 (5.5–16)	15 (9–22)	7 (3–5)
STp (9)	5 (2.5–8.5)	10 (7.5–18.5)	5 (5–23)
Children			
STh (17)	5 (2–9.5)	1 (1–2.5)	4 (3–5)
STp (11)	3 (2–12)	1 (1–3)	3 (3–3)

^a Values are given as medians, with 25th to 75th percentiles in parentheses. *P* values (STh versus STp using Mann-Whitney test) are as follows: duration, 0.26 (travelers) and 1 (children); symptoms, 0.54 (travelers) and 0.74 (children); unformed stools, 0.014 (travelers) and 0.063 (children).

^b For travelers, the symptom score is the sum of maximum intensity ratings for gastrointestinal and general symptoms reported by a subject anytime during the episode. For children, five clinical symptoms were recorded as present or absent (the severity of each symptom was not recorded): nausea, vomiting, abdominal pain, fever, and excretion of mucus.

TABLE 2. Relative distribution of ST genotypes in ST-positive ETEC strains isolated from the stools of travelers to Guatemala and Mexico with diarrhea or in nondiarrheal routine samples from travelers

ST genotype	Number (%) of strains isolated from:	
	Diarrhea cases ($n = 30$)	Routine specimens ($n = 22$)
STh	16 (53)	10 (45)
STp	13 (43)	11 (50)
STh STp	1 (3)	1 (5)

FIG. 2. STp (A) and STh (B) PCR of ETEC strains from Guatemala. Lane Mw, molecular weight marker. Lanes 1 to 9, ETEC isolates; lane 10, STh- and STp-positive reference strain E17018/A; lanes 11 and 12, ETEC isolates; lane 13, PCR control without DNA; lane 14, ETEC isolate; lane 15, STp (in panel A)- and STh (in panel B)-positive reference strains.

for STp in Bangladesh, and two strains were positive for both STh and STp.

A few strains were found to be negative for both STh and STp in the initial screening using boiled DNA. These strains were retested by DNA hybridization with specific probes (17) and were then found to be positive for either STh or STp. When these strains were reanalyzed by PCR with newly prepared DNA using a commercial DNA purification kit, they were found to contain the same STa gene type as determined by DNA hybridization.

The possibility that STp was associated with ST-only strains in a higher frequency than with ST/LT strains, as initial results with Guatemalan strains indicated, was investigated. This could not be corroborated when analyzing strains isolated from Egyptian and Guatemalan children with diarrhea, showing that ST-only strains were of the STh genotype in 68% and 63% of the cases, respectively, whereas a somewhat more equal distribution of the two ST genotypes was found among the ST/LT strains from both countries (Table 1) (the prevalence of STp strains was too low in Bangladesh to allow a comparison). However, when strains isolated from adult travelers to Guatemala and Mexico were analyzed, it was found that the STp genotype dominated among ST-only strains (83%), whereas it was found in low frequency in ST/LT strains (11%).

Correlation of STa genotype with severity of disease. It has been suggested that only STh-positive but not STp-positive ETEC strains are associated with diarrhea (11, 18). We therefore compared the distribution of STh and STp among STa-positive ETEC strains isolated from travelers with diarrhea (in whom ETEC had been identified as the single pathogen) and in routine samples (not associated with diarrhea episodes) from the same group of travelers (Table 2). These analyses showed that 53% of the strains from the diarrhea samples comprised the STh genotype and 43% the STp genotype, while the ETEC strains isolated from nondiarrhea samples were of the STh genotype in 45% of the cases and of the STp genotype in 50% of the cases. Both sets of strains contained one STh/STp-positive isolate each. Thus, no association between a specific STa genotype and disease was found. This was further analyzed by comparing the severity of disease caused by ETEC strains with STh and STp genotypes in travelers to Guatemala and Mexico and in Guatemalan children. Data regarding duration of disease, severity of symptoms, and number of diar-

real stools in cases where data were available, were analyzed (Table 3). No significant difference in duration of the disease or symptoms between STh and STp was found. The number of unformed stools per day was slightly higher in both travelers and children infected by STh strains. These results further strengthen the conclusion that there are no major differences in the disease outcome between diarrhea caused by ETEC positive for STh or STp.

DISCUSSION

An important objective of this study was to evaluate whether ETEC strains producing both subtypes of STa, i.e., STh or STp, could be associated with diarrhea or found as the single pathogen in diarrhea stools. Thus, it had recently been reported that ETEC strains producing STp alone or in combination with LT are not associated with diarrhea in children either in Israel or in Guinea-Bissau (11, 18). To evaluate whether this is a general phenomenon in different parts of the world and in different categories of subjects, we have analyzed a large number of ETEC strains isolated in recent or ongoing studies of ETEC diarrhea and infection in children in Asian, African, and Latin American countries and also in travelers to Latin America.

To enable these studies, we wanted to use sensitive and highly specific PCR methods to allow identification of the genes for STh and STp, respectively. Thus, in initial studies, different sets of primers that had been described in the literature (16, 22) for identification and differentiation of the two genotypes of STa were tested on a number of ETEC reference strains expressing STh or STp, as recently determined by gene probe assays (18). When the multiplex PCR, described by Stacy-Phipps et al. (16) to allow simultaneous detection of STh and STp, was used, we could amplify neither the STh nor the STp gene in our set of reference strains with defined STa genotypes. This may be due to differences in preparing DNA for PCR analyses, since we used supernatants from boiled bacteria as opposed to extensively purified DNA. However, the STp PCR described by Woodward et al. (22) that has earlier been used to characterize animal *E. coli* strains from a variety of sources worked well in our studies when tested on a number of reference strains. However, since we could not identify any previously described PCR that specifically identified STh-positive strains, we established a STh PCR method with a new set of primers, after having tested a series of newly selected primers. When using crude DNA from boiled bacterial cells and the STp PCR method (22) together with our newly developed PCR for STh, a majority of the STa-positive strains included in this study could be identified as STh, STp, or STh/STp. In the few cases where no PCR products were obtained, the samples had been stored for a longer period, and when these strains were retested using purified DNA, they were then found to be positive for either STh or STp. This emphasizes the importance of using freshly prepared DNA samples from boiled bacteria.

Our findings clearly show that STa-only as well as ST/LT ETEC strains producing either STh or STp are associated with diarrhea in children in countries of endemicity. Thus, we could show almost as high frequency of STp as of STh ETEC strains in diarrhea stools from children in Guatemala and Egypt. The lower frequency of STp strains in diarrhea stools in Bangladesh

is in agreement with similar low frequencies of STp ETEC isolated from environmental samples in Bangladesh (1). Similarly, we found that a high proportion of ETEC strains isolated from travelers with diarrhea, and with ETEC as the only pathogen, produced STp. At variance with the findings for the children's strains which had similar distributions of STh and STp among ST-only and ST/LT ETEC, there was a higher proportion of STp among ST-only and a lower proportion of STp among ST/LT isolated from the travelers with diarrhea. However, these comparisons are based on relatively small numbers of strains and will be further evaluated in future ETEC disease burden studies both in areas of endemicity and in travelers.

The different findings in Guinea-Bissau and Israel compared to those in Egypt or Guatemala may be explained by a number of different factors. One possibility may be that they are related to usage of different methods, i.e., DNA-DNA colony hybridization versus PCR. Although in the laboratory in Göteborg, there has been a very good agreement in identification of STh and STp by using the same gene probes as in the Guinea-Bissau studies (8, 18) and the PCR methods and primers used in these studies (Å. Sjöling, G. Wiklund, and A.-M. Svennerholm, unpublished data). Other explanations may be related to study design differences and the definition of a diarrhea episode. Thus, the study in, e.g., Guinea-Bissau was a birth cohort study based on active surveillance, and the children were categorized as having diarrhea or no diarrhea by the caretaker, i.e., a change in stool consistency to the liquid side combined with an increase in stool frequency (8). A similar approach was used for collection of the Egyptian strains used in our study which were obtained from children with diarrhea by active, in-home surveillance for diarrhea for participants in a vaccine trial, but nevertheless, the difference in STp prevalence remains. The studies in Guatemala/Mexico and Bangladesh, on the other hand, were based on passive surveillance, i.e., children or travelers with diarrhea were seeking care for their symptoms and all strains had been isolated from patients with three or more loose or liquid stools in 24 h and ETEC was only recognized enteropathogen. However, the study design is a less likely explanation, since most of the diarrhea cases in our studies were probably more severe than in the Guinea-Bissau study, yet we identified ETEC producing STp alone, or in combination with LT, in a high proportion of diarrhea stools. Yet another and more likely explanation is that the results from Guinea-Bissau and Israel are based on the determination of odds ratios between "diarrhea" and infection, whereas we have determined association between ETEC as the only recognized pathogen in 98% of the cases and diarrhea. Furthermore, when we compared the duration and severity of disease in children and travelers infected with ETEC expressing STh or STp, no significant difference was found. A slightly increased frequency of loose stools in both travelers and children infected with STh compared to STp was noted. However, this comparison was based on a small number of cases and, thus, we cannot exclude that, on a population basis, there may be a stronger association between diarrhea and STh- than STp-producing ETEC strains, but this has to be assessed in well-controlled prospective case control studies in which an established definition of diarrhea is used.

In conclusion, we have established highly specific PCR

methods that allow discrimination of STh and STp. When using these methods, we have shown that ETEC strains isolated as the only pathogen from diarrhea stools may express STp and STh at almost similar frequencies in specimens collected from different geographic areas and categories of patients and with similar disease outcomes.

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