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Pathogenicity and Phenotypic Characterization of Enterotoxigenic *Escherichia coli* Isolates from a Birth Cohort of Children in Rural Egypt

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Enterotoxigenic *Escherichia coli* (ETEC) has consistently been the predominant bacterial cause of diarrhea in many birth cohort- and hospital-based studies conducted in Egypt. We evaluated the pathogenicity of ETEC isolates in a birth cohort of children living in a rural community in Egypt. Between 2004 and 2007, we enrolled and followed 348 children starting at birth until their second year of life. A stool sample and two rectal swabs were collected from children during twice-weekly visits when they presented with diarrhea and were collected every 2 weeks if no diarrhea was reported. From routine stool cultures, five *E. coli*-like colonies were screened for ETEC enterotoxins using a GM1 enzyme-linked immunosorbent assay (ELISA). The isolates were screened against a panel of 12 colonization factor antigens (CFAs) by a dot blot assay. A nested case-control study evaluated the association between initial or repeat excretion of ETEC and the occurrences of diarrhea. The pathogenicity of ETEC was estimated in symptomatic children compared to that in asymptomatic controls. ETEC was significantly associated with diarrhea (crude odds ratio, 1.37; 95% confidence interval [CI], 1.24 to 1.52). The distribution of ETEC enterotoxins varied between the symptomatic children (44.2% heat-labile toxin [LT], 38.5% heat-stable toxin [ST], and 17.3% LT/ST) and asymptomatic children (55.5% LT, 34.6% ST, and 9.9% LT/ST) ($P < 0.001$). The CFAs CFA/I ($n = 61$), CS3 ($n = 8$), CS1 plus CS3 ($n = 24$), CS2 plus CS3 ($n = 18$), CS6 ($n = 45$), CS5 plus CS6 ($n = 11$), CS7 ($n = 25$), and CS14 ($n = 32$) were frequently detected in symptomatic children, while CS6 ($n = 66$), CS12 ($n = 51$), CFA/I ($n = 43$), and CS14 ($n = 20$) were detected at higher frequencies among asymptomatic children. While all toxin phenotypes were associated with diarrheal disease after the initial exposure, only ST and LT/ST-expressing ETEC isolates ($P < 0.0001$) were associated with disease in repeat infections. The role of enterotoxins and pathogenicity during repeat ETEC infections appears to be variable and dependent on the coexpression of specific CFAs.

Enterotoxigenic *Escherichia coli* (ETEC)-associated diarrhea is the most common bacterial diarrhea affecting both children <5 years of age and adults living in developing countries, as well as travelers to these areas (1, 2). ETEC causes an estimated 280 million diarrheal episodes and >380,000 deaths annually (2). In Egypt, ETEC is a leading bacterial cause of diarrhea in children and visitors, as evidenced in traveler, community (birth cohorts), and hospital-based studies (3–7). The incidence of ETEC-associated diarrhea among children <3 years of age in rural Egypt was estimated to be 1.5 episodes per child-year, which ranks among the highest rates reported (8).

ETEC isolates express a combination of colonization factor antigens (CFAs) and enterotoxins; these virulence factors are necessary for pathogenesis, and their genes are primarily carried on plasmids. ETEC adheres to the intestinal mucosa using the CFAs, and after binding, it secretes a heat-labile toxin (LT) and/or a heat-stable toxin (ST). The LT induces diarrhea through binding to intracellular adenyl cyclase, leading to increased cyclic AMP levels, and the ST induces diarrhea through increasing intracellular cyclic GMP levels (9–11). The phenotypic characteristics of ETEC are different between communities (12) and can change over time (13). Understanding the association between ETEC phenotype and clinical virulence may be important for vaccine development efforts focusing on a toxin- and/or a colonization factor-based vaccine. Our objective was to identify the ETEC phenotypic characteristics that are associated with diarrhea during initial and repeat ETEC infections in children living in a rural community in Egypt by using a birth cohort study.

MATERIALS AND METHODS

A birth cohort study was conducted between 2004 and 2007 in a rural community in the Nile Delta of Egypt. Three hundred forty-eight children were enrolled shortly after birth and were followed for 2 years. The study was designed to include asymptomatic sampling to better understand the dynamics of pathogenic ETEC shedding in the context of understanding transmission dynamics, the host-agent relationship, and potential vaccine

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impacts (14). Therefore, during twice-a-week home visits, if the child had diarrhea, fecal specimens and two rectal swabs were collected. In addition, a fecal specimen and two rectal swabs were collected every 2 weeks during home visits regardless of the child's symptoms. The rectal swabs were placed in both Cary-Blair (CB) and buffered glycerol saline (BGS) transport media. All specimens were transported immediately on ice packs to the Abu Homos field laboratory. The rectal swabs were refrigerated at 2 to 8°C; stool samples were aliquoted in 3 cryotubes (2 ml each) and stored at −20°C. Within 3 days of collection, the specimens were sent to the U.S. Naval Medical Research Unit No. 3, Cairo, Egypt (NAMRU-3), where the rectal swabs were evaluated and the stool samples were transferred to a −70°C freezer for long-term storage. Epidemiological and clinical data were recorded on a structured pretested questionnaire. Stool cultures were performed using standard microbiological methods to detect *Campylobacter* spp., *E. coli*, *Salmonella* spp., *Shigella* spp., and *Vibrio* spp. (15). Rotavirus was detected using the Rotaclone enzyme-linked immunosorbent assay (ELISA) (Premier Rotaclone; Meridian Bioscience, Cincinnati, OH, USA) according to the manufacturer's instructions. The rectal swabs were plated on MacConkey lactose medium, and a GM1-ganglioside ELISA was used to evaluate five lactose-positive colonies for LT and ST expression (16–18). ETEC isolates were typed for CFA expression with a dot blot assay using a panel of 12 monoclonal antibodies against CFA/I, CS1 to CS8, CS12, CS14, and CS17 (19–21).

The incidence rates were calculated by dividing the number of episodes by the number of person-years at risk (total person-years of follow-up minus the duration of diarrheal episodes).

A nested case-control study was used to evaluate the association between the initial excretion of ETEC and the occurrence of diarrhea (pathogenicity). An age- and season-matched asymptomatic control was defined as a child without a loose or liquid stool for a period that included the day of the routine visit and 3 days before or after the visit. Cases were defined as patients with episodes of diarrhea, defined as loose stools that began after at least three consecutive nondiarrheal days and ending when followed by at least three consecutive nondiarrheal days, detected during the twice-weekly visits. Children with a history of having ETEC detected in their stool were excluded from selection as either a case or a control. Only the first fecal specimen obtained during each episode was included in this analysis to avoid bias from repeated fecal sampling during a single diarrheal episode.

Univariate analysis identified confounding variables as predictors of ETEC incidence to include age, gender, season, maternal education, history of being breastfed, and household crowding. An assessment of household crowding was based on the number of residents per sleeping room and was categorized by tertiles as low (0.5 to <3.5 persons per room), medium (≥3.5 and <4.75 persons per room), or high (≥4.75 persons per room).

Exposure to ETEC in cases relative to controls was expressed as an odds ratio (OR). All episodes of diarrhea (symptomatic) or episodes that correlated with asymptomatic infection for a subject subsequent to the initial ETEC infection were dropped when estimating the relative odds of exposure. To control for confounding factors, ORs were obtained from a multivariate logistic regression model that used generalized equations to adjust for the independence of multiple observations for individual subjects. To ensure that the associations reflected concurrent comparisons of episodes and controls, an indicator variable for a calendar quarter (3-month intervals) was also fitted to the model. *P* values and 95% confidence intervals (CIs) for the ORs were calculated in a two-tailed fashion from the coefficients and the standard error (SE) of these coefficients. SAS software (version 9.1; SAS Institute, Inc., Cary, NC, USA) was used for all analyses.

Another case-control analysis was performed to evaluate the pathogenicity of recurrent infections in patients who had multiple episodes of diarrhea detected during the cohort study. Control subjects were children selected during the every-2-weeks routine visits who did not have diarrheal episodes within 3 days of the survey visit. Cases and controls were adjusted to the previously mentioned confounders by multivariate logistic regression analysis. ETEC pathogenicity was assessed by comparing the prevalence of fecal excretion of ETEC in the case patients and in control subjects. These associations were expressed as ORs. Because a single diarrheal episode could have been evaluated on the basis of multiple rectal swab specimens if the episode continued over several visits, only the results of the first rectal swab of each case were considered in the comparison with the controls, from whom only one rectal swab was obtained.

The study protocol (NAMRU3.2003.0011, IRB no. 145) was approved by the U.S. Naval Medical Research Unit No. 3 Institutional Review Board in compliance with all applicable federal regulations governing the protection of human subjects.

RESULTS

ETEC was the most common pathogen detected among the children in the study. During the 2-year study period, ETEC was isolated from 18.9% (756/4,001 samples) of the diarrheal episodes and 13.6% (1,299/9,539 samples) of the stool samples from asymptomatic children collected during the twice-weekly visits. Six hundred thirty-two episodes were due to ETEC as the sole pathogen, with an incidence rate of 1.24 episodes per child-year. ETEC infection was significantly associated with diarrhea, with a relative risk of 1.37 (95% CI, 1.24 to 1.52). Other enteric pathogens that were also isolated from stool during diarrheal episodes were *Campylobacter* spp. ($n = 319$ [8%]), *Shigella* spp. ($n = 48$ [1.2%]), *Salmonella* spp. ($n = 10$ [0.2%]), and rotavirus ($n = 216$ [5.4%]), whereas the organisms isolated from stools of asymptomatic children in lower frequencies were *Campylobacter* spp. ($n = 691$ [7.2%]), *Shigella* spp. ($n = 20$ [0.2%]), and *Salmonella* spp. ($n = 7$ [0.1%]). We did not test for rotavirus in asymptomatic children.

Of the 2,055 ETEC isolates, 318 (15.5%) were isolated from children <6 months of age. Additionally, in this age group, ETEC was isolated from only 46.5% (148/318) of the children reporting diarrhea. The frequency of isolation of ETEC from stool samples increased to 28.2% (579/2,055) during the second 6 months of life and remained at this level during the second year of life. However, the likelihood of isolating ETEC in the samples from children without diarrhea (asymptomatic/symptomatic) increased with the age of the children, ranging from a ratio of 1.1 for children <6 months of age to 2.5 in children 18 to 23 months of age (see Fig. S1 in the supplemental material).

Among the ETEC-associated diarrheal episodes, 334 ETEC diarrheal episodes (44.2%) were associated with LT-expressing ETEC, 291 (38.5%) were associated with ST-expressing ETEC, and 131 (17.3%) were associated with isolates that produced both enterotoxins (see Table S1 in the supplemental material). The incidence of ETEC-associated diarrhea was the highest (1.8 episode per child-year) in the second 6 months of life and then decreased in the older age groups. ST-expressing ETEC-associated diarrhea ($n = 174$) was most common in the first year of life (60% [174/291]), but LT-expressing ETEC-associated diarrhea ($n = 183$) was more common in the second year of life (55% [183/334]). CFAs were identified in 33% (252/756) of all ETEC-associated diarrhea cases (see Table S2 in the supplemental material). CS7 ($n = 18$) and CS17 ($n = 9$) were the most frequently expressed CFAs with LT-expressing ETEC, CFA/I ($n = 48$) and CS6 ($n = 38$) were the most commonly associated CFAs with ST-expressing ETEC, and CS3 ($n = 31$), CS6 ($n = 19$), CFA/I ($n = 12$), and CS14 ($n = 11$) were the most common in LT/ST-expressing ETEC strains (see Table S2 in the supplemental material).

Among asymptomatic children, LT-expressing ETEC was detected in 56% (721/1,299) of the samples, while ST- and LT/ST-expressing ETEC was detected in 35% (449/1,299) and 10% (129/1,299) of the samples, respectively (see Table S1 in the supplemental material). The number of asymptomatic ETEC infections increased steadily from 170 infections in the age group of <6 months to 422 infections in the age group between 18 and 23 months. LT-expressing ETEC infection was the most common asymptomatic infection during the whole follow-up period, increasing from 45.3% of all asymptomatic ETEC infections in the age group <6 months to 61% in the group between 18 and 23 months of age. However, the incidence of ST-expressing ETEC decreased from a high of 44.7% in the age group of <6 months to 27% in the age group between 18 and 23 months. A CFA was detected in only 19% (240/1,299) of the ETEC strains isolated from healthy children; 33% (146/449) of the ST-expressing ETEC, 39% (50/129) of LT/ST-expressing ETEC, and 6% (44/721) of LT-expressing ETEC had a phenotypically detectable CFA. CS17 ($n = 11$) and CS12 ($n = 15$) were the most frequent CFAs associated with LT-expressing ETEC isolates; CFA/I ($n = 39$), CS6 ($n = 58$), CS12 ($n = 18$), and CS14 ($n = 16$) were associated with ST-expressing ETEC, while LT/ST-expressing ETEC displayed mainly CS12 ($n = 18$), CS3 ($n = 14$), and CS6 ($n = 10$) (see Table S2 in the supplemental material).

Pathogenicity of first documented ETEC infection. The phenotypic characteristics associated with ETEC isolated from the symptomatic children and asymptomatic controls were compared. Overall, the isolation of any ETEC phenotype was associated with the presence of diarrheal symptoms (OR, 1.93; 95% CI, 1.46 to 2.56) (see Table S3 in the supplemental material). The risk of ETEC infection was significantly associated with diarrhea when CFAs were detected (OR, 2.16; 95% CI, 1.62 to 2.89). An analysis by enterotoxin phenotype revealed that the initial infection with ETEC-expressing LT (OR, 1.64; 95% CI, 1.09 to 2.47), ST (OR, 1.81; 95% CI, 1.17 to 2.80), or LT/ST (OR, 2.45; 95% CI, 1.03 to 5.82) was significantly associated with diarrheal symptoms. Initial infection with ETEC-expressing ST was associated with diarrhea risk either in the presence (OR, 1.88; 95% CI, 1.18 to 2.98) or absence (OR, 1.43; 95% CI, 1.02 to 2.01) of a detectable CFA. Initial infection with LT-expressing ETEC increased diarrhea risk when a CFA was detected; however, the association was not statistically significant (OR, 1.88; 95% CI, 0.97 to 3.65) (see Table S3 in the supplemental material).

Pathogenicity of repeated ETEC infection. We compared the pathogenicity of repeated infections in symptomatic cases and controls (see Table S4 in the supplemental material); overall, ETEC infection was associated with diarrhea in all age groups (OR, 1.37; 95% CI, 1.24 to 1.52). With the exception of the group between 18 and 23 months of age (OR, 3.27; 95% CI, 1.50 to 7.13), LT-expressing ETEC infections were not associated with diarrhea. During the first 6 months of age, LT/ST-expressing ETEC was associated with diarrhea when CFAs were detected (OR, 2.79; 95% CI, 1.20 to 6.46) or not detected (OR, 2.41; 95% CI, 1.3 to 5.66). In the subsequent 6-month age groups, LT/ST-expressing ETEC was associated with diarrhea when CFAs were detected (6 to 11 months [OR, 4.07; 95% CI, 2.16 to 7.67], 12 to 17 months [OR, 4.79; 95% CI, 2.57 to 8.93], and 18 to 23 months [OR, 2.48; 95% CI, 1.36 to 4.53]) (see Table S4 in the supplemental material). For ST-expressing ETEC, the detection of CFAs was associated with diarrhea in children 6 to 11 months of age (OR, 2.11; 95% CI, 1.43 to 3.09) and 18 to 23 months of age (OR, 2.85; 95% CI, 1.77 to 4.57).

Toxin colonization factor combinations. There were some notable phenotypic combinations of toxins and specific CFAs expressed by ETEC isolates associated with diarrhea after adjusting for potential confounders. ETEC isolates that expressed LT and CS7, ST and CFA/I or CS14, or LT/ST with CFA/I, CFA/II, CS5, or CS7 were all associated with diarrhea (see Table S5 in the supplemental material). The most pathogenic of these combinations was LT/ST with CS7, which was 10 times more likely to be associated with diarrhea. On the other hand, the combination of the LT/ST with CS12 was found to be significantly associated with asymptomatic controls compared to diarrhea cases (adjusted relative risk [RRa], 0.33; 95% CI, 0.11 to 0.92).

Independent of enterotoxin type, some CFA groups or individual CFAs were pathogenic (see Table S6 in the supplemental material). The CFA that was most frequently associated with pathogenicity was CS7, which, when detected, was 6 times more likely to be associated with diarrhea. CFA/I, which was the most frequently expressed CFA in both symptomatic and asymptomatic children, was significantly associated with diarrhea (RRa, 2.82; 95% CI, 1.87 to 4.27). Other CFAs, including CS3 and CS6, were significantly associated with diarrhea when expressed alone (RRa, 2.37 and 1.62, respectively). However, the risk of diarrhea increased when CS3 or CS6 was combined with other CFAs, for example, CS3 combined with either CS1 (RRa, 4.18) or CS2 (RRa, 3.37) or CS5 detected with CS6 (RRa, 3.21). Children infected with ETEC expressing CS14 were 2.59 times more likely to develop ETEC-associated diarrhea (95% CI, 1.58 to 4.24).

DISCUSSION

One of the important advantages of cohort studies is the ability to characterize the pathogenic and virulence properties of the infecting pathogen. The current study showed that ETEC was recovered very early in life from healthy and sick children. The recovery of ETEC from stool samples peaked in children from 6 to 11 months of age (28%) and maintained this frequency for the duration of the study. However, we also noted that the isolation of ETEC from stool was not indicative of illness, as evidenced by the ratio of ETEC isolates recovered from healthy children to those from sick children, which increased from 1.1 to 2.5 by the end of the study. Our findings are consistent with other studies that have reported a declining incidence of ETEC diarrhea with age and lower ratios of ETEC infections in symptomatic to asymptomatic children with increasing age (22). Studies have shown that natural protection against ETEC-associated diarrhea emerges in children who are exposed to repeated infections (23–27); however, natural protection does not prevent ETEC colonization.

The incidences of ETEC enterotoxin phenotypes differed from those in previously published cohorts and hospital-based studies conducted in Egypt (4, 28–31). In the current cohort, ETEC isolates expressing LT were most commonly isolated, followed by ETEC isolates expressing ST and LT/ST. A previous cohort study in Egypt, which evaluated children <3 years of age, reported that 61% of the ETEC isolates expressed ST, only 26% expressed LT, and 12% expressed both toxins (30). In contrast, the distribution of expressed CFAs was similar to that of another cohort study from Egypt (30); the most common CFA phenotypes among symptomatic children were CFA/I, CS6, CS3, and CS14. This result is also consistent with those of other studies (25, 32); ETEC

strains expressing a wide range of CFA were found to be associated with diarrhea, including CFA/I, CFA/II (CS1 to CS3), CSA/IV (CS4 to CS6), CS7, and CS14. Some CFA-enterotoxin combinations were associated with diarrhea, such as CS7 with LT; this combination was also found with ETEC-associated diarrhea in previous cohort studies (33, 34). These findings are of interest, given that current colonization factor-based vaccine approaches do not consider CS7 and CS14 (these factors cross-react immunologically with CS5 and CFA/I, respectively), which may be important causes of pediatric disease (35). Other CFA-toxin phenotype combinations, such as CFA/I-ST and CS5 LT/ST alone, were found in our study to be associated with diarrhea, as well as in a comparable cohort study (27). On the other hand, the combination of LT/ST and CS12 was not found to be associated with diarrhea. It is worth mentioning that these data indicate that pathogenicity may be increased when multiple CFAs are expressed (e.g., CS1/CS3 and CS5/CS6) compared to those expressing a CFA monotype, particularly in the CFA/I and CFA/IV phenotypes. Thus, vaccine approaches directed toward anti-CFA immunity may need to include a broader array of CFAs to ensure coverage.

The case-control studies reported here identified the pathogenicity of all ETEC enterotoxins during initial ETEC infection. However, on subsequent infections, ETEC strains that secreted ST and/or LT/ST enterotoxins were more likely to be associated with diarrhea, while LT-only infections were not. The decreased pathogenicity of LT-expressing ETEC isolates has been reported previously in a study from Egypt (14), as well as in others (23). However, it was reported that LT-expressing ETEC isolates may cause severe diarrhea, given that they were isolated from patients with severe dehydrating diarrhea (25).

ETEC isolates recovered from diarrheal cases were positive for CFAs at a higher frequency than were samples from healthy children; however, the proportion of the CFAs detected was much lower than that reported from another study in Bangladesh (27). The important role of CFAs was obvious in the pathogenicity of ETEC isolate-associated diarrhea; when combined with the expressed enterotoxins, the odds of inducing diarrhea increased in subsequent infections. Even when the pathogenicity associated with ETEC-expressing CFAs was studied alone, several CFAs were highly associated with ETEC-associated diarrhea.

Limitations. One of the main findings of this study is the low frequency of ETEC isolates expressing a known colonization factor (33.3% in symptomatic episodes and 18.5% in asymptomatic samples). The phenotypic method used in this study underestimates CFA detection for a couple reasons, including the limited number of CFAs that have monoclonal antibodies which can be used in the assay and the requirement for the expression of the fimbriae in the *in vitro* environment. It is well known that the production of CFAs can be variable due to genetic defects in the genes required for expression (27) and the composition of bacterial growth media and growth conditions; this is particularly true for CS21, which requires unique growth conditions (36). A number of studies have reported that genotypic methods offer certain advantages over phenotypic methods (18, 37). These studies suggest that there is increased sensitivity and specificity of CFA detection using molecular tools. Future studies should plan to utilize molecular diagnostic techniques to better determine the incidence of ETEC and its associated CFAs on the same set of samples. Finally, understanding the association of ETEC phenotype with moderate to severe disease is important. Due to the design of this cohort (active surveillance) and nested case-control study, symptomatic cases were predominately mild in severity. Additional studies are needed to determine which CFA-toxin phenotypes may be attributed to more severe disease. Such information is also critical for the development of a vaccine design with maximal impact.

Conclusion. There is a clear need for the development of an effective ETEC vaccine (24) for children living in developing countries and for travelers to these countries. A number of vaccine approaches are currently being pursued with a combined toxin-CFA approach (34). These approaches include whole-cell, live-attenuated, or killed and fimbrial subunit approaches, which are combined with anti-LT immunity. Ideally, a vaccine needs to have broad coverage and durable immunity to be an effective solution for reducing the burden of disease among children at risk globally. These data provide additional important information as to the potential coverage of CFA–toxin-based vaccine approaches.

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