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Phenotypic Diversity of Enterotoxigenic *Escherichia coli* Strains from a Community-Based Study of Pediatric Diarrhea in Periurban Egypt

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No past studies of diarrhea in children of the Middle East have examined in detail the phenotypes of enterotoxigenic *Escherichia coli* (ETEC) strains, which are important pathogens in this setting. During a prospective study conducted from November 1993 to September 1995 with 242 children under 3 years of age with diarrhea living near Alexandria, Egypt, 125 episodes of diarrhea were positive for ETEC. ETEC strains were available for 98 of these episodes, from which 100 ETEC strains were selected and characterized on the basis of enterotoxins, colonization factors (CFs), and O:H serotypes. Of these representative isolates, 57 produced heat-stable toxin (ST) only, 34 produced heat-labile toxin (LT) only, and 9 produced both LT and ST. Twenty-three ETEC strains expressed a CF, with the specific factors being CF antigen IV (CFA/IV; 10 of 23; 43%), CFA/II (5 of 23; 22%), CFA/I (3 of 23; 13%), PCFO166 (3 of 23; 13%), and CS7 (2 of 23; 9%). No ETEC strains appeared to express CFA/III, CS17, or PCFO159. Among the 100 ETEC strains, 47 O groups and 20 H groups were represented, with 59 O:H serotypes. The most common O serogroups were O159 (13 strains) and O43 (10 strains). O148 and O21 were each detected in five individual strains, O7 and O56 were each detected in four individual strains, O73, O20, O86, and O114 were each detected in three individual strains, and O23, O78, O91, O103, O128, and O132 were each detected in two individual strains. The most common H serogroups were H4 (16 strains), 12 of which were of serogroup O159; H2 (9 strains), all of which were O43; H18 (6 strains); H30 (6 strains); and H28 (5 strains); strains of the last three H serogroups were all O148. Cumulatively, our results suggest a high degree of clonal diversity of disease-associated ETEC strains in this region. As a low percentage of these strains expressed a CF, it remains possible that other adhesins for which we either did not assay or that are as yet undiscovered are prevalent in this region. Our findings point out some potential barriers to effective immunization against ETEC diarrhea in this population and emphasize the need to identify additional protective antigens commonly expressed by ETEC for inclusion in future vaccine candidates.

Enterotoxigenic *Escherichia coli* (ETEC) is a leading cause of pediatric diarrhea in the developing world and is also an important cause of diarrhea in adult travelers to these regions (5, 7, 8, 17). On an annual basis, ETEC strains have been estimated to cause 400 million episodes of diarrhea in children under age 5, resulting in 700,000 deaths (39). As a cause of traveler's diarrhea, it has substantial impacts in terms of both morbidity and economic consequences (32).

Two virulence attributes that characterize ETEC are the capacity to adhere to the small intestinal surface and to secrete enterotoxins. Over 20 distinct, human-specific ETEC adhesins or colonization factors (CFs) have been described, most but not all of which constitute surface-exposed fimbriae or fibrillae (for reviews, see references 10 and 21). In many geographic areas, the most common CFs individually expressed by ETEC strains are CF antigen I (CFA/I), CFA/II, which is composed of coli surface antigen CS3 alone or in combination with CS1 or CS2 (16, 34), and CFA/IV, which is composed of CS6 alone or in combination with CS4 or CS5 (38). Besides CFs, ETEC strains elaborate one or both of two well-defined enterotoxins: a heat-labile toxin (LT), which is structurally and functionally similar to cholera toxin (13), and a heat-stable toxin (ST), which is a low-molecular-weight, poorly immunogenic peptide (14, 20). ETEC strains also express somatic (O) and flagellar (H) antigens on the cell surface, differentiation of which forms the basis for serotype classification of *E. coli* (for a review, see reference 28). While not specifically implicated as virulence determinants, certain O and H antigens have typically been associated with ETEC, while others are found in association with other diarrheagenic categories of *E. coli* as well as pathogenic *E. coli* strains that cause urinary tract infections and neonatal meningitis.

Both LT and individual CFs have been implicated as protective antigens and have accordingly become the focus of

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ETEC vaccine development efforts (2, 33). As specific vaccine candidates advance to field evaluation, it becomes imperative to characterize the extent of phenotypic diversity of ETEC with respect to these virulence factors to permit predictions of the theoretical extent of vaccine coverage. Since the relative distribution of ETEC toxin and CF phenotypes can vary from one geographic location to another, specific data are needed for each site where vaccine testing is contemplated.

Past studies in the Middle East and Northern Africa have indicated that ETEC strains are important causes of early childhood diarrhea in this region (30, 44). A limitation of these studies was that the ETEC isolates from children with diarrhea were tested only for enterotoxin type. In a recent population-based study conducted with individuals living on the outskirts of Alexandria, Egypt, we corroborated the high incidence of ETEC-associated diarrhea in Egyptian children under 3 years of age (1). Here we present detailed data on the distribution of toxin and CF phenotypes as well as the results of serotype analysis of diarrhea-associated ETEC strains isolated from this cohort of children.

MATERIALS AND METHODS

Bacterial strains. ETEC strains were originally isolated from stool samples obtained from children with diarrhea (birth to 35 months of age) in the village of Abees, Alexandria Governorate, Egypt, in a prospective study conducted between November 1993 and September 1995. The guardians of each subject gave voluntary, informed consent for participation prior to enrollment in this study. The design and methods of this study are presented in detail elsewhere (1). Over the course of the study 125 episodes of ETEC diarrhea were detected. ETEC isolates from 98 of these episodes were available for further analysis.

A defined set of criteria was used to select ETEC strains for the analyses presented in this report. First, the ETEC isolates associated with each episode of diarrhea were identified. If the ETEC isolates from a given diarrheal episode were identical in terms of enterotoxin type, they were assumed to represent a single clonal strain of ETEC. If any isolate from an episode expressed a CF, it was selected as the representative strain; otherwise, a toxin-positive, CF-negative isolate was selected. In the event that a child suffering from diarrhea was infected with ETEC isolates of different enterotoxin and CF phenotypes, single isolates that represented these different phenotypes were selected.

The ETEC strains used as controls for enterotoxin and CF expression in this study were E259093 (CFA/I), E1392-75 (CS1), E278485 (CS2), VM73494 (CS3), E344206 (CFA/III), E11881/9 (CS4), E170181/1 (CS5), VM75688 (CS6), E29101A (CS7), E20738A (CS17), E350C/A (PCFO159), E3476A (PCFO166), 286C2 (LT), ST64111 (ST), 258909-3 (LTST), and HS4, a commensal strain isolated from a human volunteer. All strains were maintained at −70°C in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) containing 15% glycerol.

Enterotoxin, CFA, and serotype analysis. The GM1 enzyme-linked immunosorbent assay for LT and STa was performed as described previously (36). Strains that produced LT and/or STa were subsequently tested for CF expression by a colony dot blot assay with monoclonal antibodies against CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS17, PCFO159, PCFO166, and CFA/III (3, 24, 40). All strains were serotyped with absorbed polyclonal antibodies by the reference serology laboratory at the Universidad Nacional Autonoma de Mexico.

RESULTS

Over the course of a prospective, community-based study of diarrhea in children under 3 years of age in Abees, Egypt, 242 children with diarrhea were examined for detection of bacterial etiologies, including ETEC (1). During this study, 125 episodes of ETEC-associated diarrhea were detected. ETEC isolates from 98 of these episodes had been preserved, and from these isolates representative ETEC strains were selected. A single ETEC strain (with a distinct toxin and CF profile) was selected from among the isolates cultured from 96 of these episodes, while two phenotypically distinct ETEC strains each were selected from among the isolates cultured from two additional episodes of phenotypically mixed ETEC infections, giving a total of 100 strains that were selected for further analysis.

Of these strains, 57 produced ST only, 34 produced LT only, and 9 produced both LT and ST (Table 1). For the two episodes from which dual ETEC pathogens were isolated, one episode was associated with excretion of an ETEC strain that was positive for ST only and CS6 positive and one that was positive for LT only and CF negative and the other was associated with excretion of an ETEC strain that was positive for LT only and CF negative and one that was positive for ST only and CF negative.

TABLE 1. Relationship between CFA and enterotoxin expression

CFA	No. of strains producing the following:			
	LT	ST	LT and ST	Total
CFA/I	0	0	3	3
CFA/II	0	5	0	5
CFA/IV	1	8	1	10
Others	1	2	2	5
None	32	42	3	77
Total	34	57	9	100

Overall, 23% of ETEC strains expressed 1 or more of the 12 CFs tested. The proportion of CF-positive ETEC strains varied by toxin phenotype. ETEC strains that produced LT and ST were most likely to express a CF (6 of 9; 67%), followed by strains that expressed ETEC ST only (15 of 57; 26%) and ETEC strains that expressed LT only ETEC (2 of 34; 6%). The specific CFs detected were, in descending order of frequency, CFA/IV (10 of 23; 43%), CFA/II (5 of 23; 22%), CFA/I (3 of 23; 13%), PCFO166 (3 of 23; 13%), and CS7 (2 of 23; 9%). No ETEC strains appeared to express CFA/III, CS17, or PCFO159. Of the CFA/IV-positive ETEC strains, the majority (8 of 10; 80%) expressed CS6 only, with the remainder expressing CS5 and CS6. Four of the five CFA/II-positive ETEC strains expressed CS1 and CS3, while the remaining strain expressed CS2 and CS3. Expression of CFA/I was detected only in combination with LT and ST production, whereas all CFA/II-positive ETEC strains produced ST only. All eight ETEC strains positive only for CS6 expressed ST only, while of two ETEC strains positive for CS5 and CS6, one expressed LT only and one expressed both LT and ST.

There was a remarkable diversity of individual O and H serogroups and O:H serotype combinations detected among the 100 ETEC strains (Table 2). Forty-seven O groups, 20 H groups, and 59 O:H serotypes were represented; these do not take into account those strains which were either O or H nontypeable. Sixteen O serogroups were detected in two or more ETEC strains, representing a total of 65 strains, while 31 O serogroups were detected in a single strain each, and 4 strains were either rough or O nontypeable. The most common O serogroups were O159 (13 strains) and O43 (10 strains). Serogroups O148 and O21 were each detected in five individual strains, O7 and O56 were each detected in four individual strains, O73, O20, O86, and O114 were each detected in three individual strains, and O23, O78, O91, O103, O128, and O132 were each detected in two individual strains.

Serogroups O159, O148, O20, O78, O128, O6, O8, and O15, all which have been typically associated with ETEC, were detected individually in 28 of the 100 ETEC strains. Of these, many had what would be considered a classic ETEC serotype, including O159:H4 (12 strains), O148:H28 (5 strains), O6:H16 (1 strain), and O8:H9 (1 strain). A small number of additional strains were of O serogroups that have infrequently been detected in ETEC strains, namely, O85, O88, O114, and O153 (16). Fourteen ETEC strains expressed O serogroups that have

TABLE 2. Association of toxins with CFA expression and serotype

Toxin produced	O group	H group	CFA	No. of strains
LT only	O159	H4	NEG[a]	11
	O159	H4	CFA/IV	1
	O114	H−	NEG	1
	O114	H49	CS7	1
	O91	H4	NEG	1
	O91	H?	NEG	1
	O5	H?	NEG	1
	O9	H−	NEG	1
	O21	H56	NEG	1
	O23	H15	NEG	1
	O29	H10	NEG	1
	O39	H30	NEG	1
	O43	H2	NEG	1
	O53	H18	NEG	1
	O56	H−	NEG	1
	O71	H4	NEG	1
	O88	H25	NEG	1
	O111ab	H−	NEG	1
	O118	H34	NEG	1
	O132	H16	NEG	1
	O153	H10	NEG	1
	O165	H?	NEG	1
	O?	H−	NEG	1
	OR	H4	NEG	1
LT and ST	O21	H56	NEG	2
	O128	H12	CFA/I	1
	O128	H?	CFA/I	1
	O56	H−	NEG	1
	O78	H−	PCFO166	1
	O85	H5	CFA/IV	1
	O114	H49	CS7	1
	OR	H5	CFA/I	1
ST only	O43	H2	NEG	5
	O43	H2	CFA/IV	3
	O43	H−	CFA/IV	1
	O148	H28	NEG	3
	O148	H28	CFA/IV	2
	O7	H−	NEG	3
	O7	H5	NEG	1
	O73	H18	NEG	3
	O56	H−	NEG	2
	O103	H30	CFA/II	1
	O103	H30	NEG	1
	O86	H−	PCFO166	1
	O86	H15	CFA/IV	1
	O86	H30	NEG	1
	O20	H−	NEG	1
	O20	H9	NEG	1
	O20	H34	NEG	1
	O21	H27	NEG	1
	O21	H45	NEG	1
	O1	H30	NEG	1
	O6	H16	CFA/II	1
	O8	H9	CFA/II	1
	O12	H?	CFA/II	1
	O15	H18	NEG	1
	O22	H?	CFA/II	1
	O23	H15	NEG	1
	O51	H10	NEG	1
	O54	H−	CFA/IV	1
	O62	H30	NEG	1
	O77	H18	NEG	1
	O78	H−	PCFO166	1
	O98	H25	NEG	1
	O117	H−	NEG	1
	O119	H−	NEG	1
	O125	H9	NEG	1
	O126	H11	NEG	1
	O132	H16	NEG	1
	O138	H26	NEG	1
	O159	H−	NEG	1
	O161	H4	NEG	1
	O166	H45	NEG	1
	O171	H−	NEG	1
	O?	H21	NEG	1

[a] NEG, negative.

been associated with other categories of diarrheagenic *E. coli*, such as enteropathogenic *E. coli* (O86, O119, O125, and O126), Shiga-like toxin-producing *E. coli* (O111ab and O117), and enteroinvasive *E. coli* (O29) or with uropathogenic *E. coli* (O1 and O7) (23, 27). The remaining serotypes observed have not been ascribed to any category of pathogenic *E. coli*. Those strains that possessed an ETEC-related O serogroup were more likely to express a CF (12 of 34; 35%) than were those that possessed an O group not heretofore associated with any particular category of pathogenic *E. coli* (9 of 52; 17%) or those associated with a category of pathogenic *E. coli* other than ETEC (2 of 14; 14%).

An H serogroup was detected in 74 of 100 ETEC strains, with the remainder being either nonmotile (20 strains) or H nontypeable (6 strains). The most common H serogroups were H4 (16 strains), 12 of which were of serogroup O159; H2 (9 strains), all of which were O43; H18 (6 strains); H30 (6 strains); and H28 (5 strains); strains of the last three H serogroups were all serogroup O148. The remaining 34 H typeable strains were distributed among 15 H groups; none of the H serogroups was detected in more than three strains each.

Among the more common serogroups seen, there were some notable associations or lack thereof with specific toxin and/or CF phenotypes. The majority of ETEC strains that expressed CFA/I, CFA/II, or CFA/IV were observed in combination with serotypes not usually associated with each adhesin. The few exceptions were a single O128:H12 ETEC strain that expressed CFA/I, a CFA/II strain of serotype O6:H16, and two strains of serotype O148:H28 that expressed CFA/IV. PCFO159 was not detected in any of the 13 O159:H4 strains or the single O159:H− ETEC strain. Only one of these strains expressed a CF, namely, CS5 and CS6. No CF could be detected for the one O166:H45 ETEC strain, while the three strains that expressed PCFO166 were either serotype O78:H− (two strains) or O86:H− (one strain). Two strains expressed CS7, and both of these strains were serotype O114:H49. In terms of toxin-serotype associations, all (5 of 5) O148:H28 strains and 89% (8 of 9) O43:H2 strains produced ST only, while 92% (12 of 13) serogroup O159 strains produced LT only.

DISCUSSION

This is the first study to examine in detail the phenotypic characteristics of ETEC strains associated with community-acquired pediatric diarrhea in Egypt or other countries in the Middle East. Since we and others have found a high incidence of ETEC diarrhea in young children in Egypt (1, 44), one of our underlying objectives has been to assess the suitability of such populations for use in evaluations of the efficacies of

investigational ETEC vaccines. Three findings from this study are pertinent to this aim. First, in terms of toxin profile, the majority of ETEC strains produced only ST. Second, we were unable to detect expression of an ETEC adhesin in over 75% of the strains, even though we tested for a battery of 12 described CFs. Lastly, we detected a wide array of phenotypic combinations among the ETEC strains, due particularly to the large number of serotypes seen. These observations suggest possible barriers to effective immunization against ETEC in this population, as discussed below.

It was somewhat unexpected to observe such a large assortment of serotypes among the 100 ETEC strains, many of which were detected no more than twice. Unlike the genes for toxins and CFs, which are usually plasmid encoded, the O and H biosynthetic genes are chromosomal in origin (for a review, see reference 28). This suggests a high degree of clonal diversity of disease-associated ETEC in this well-circumscribed geographic area over a relatively short period of time. Notably, the majority of the serotypes detected were not those typically associated with ETEC. O159, the predominant ETEC serogroup observed here, has been seen at a high frequency in only one other rather limited survey in the Central African Republic (9). Serotype O43:H2 was detected in 9% of ETEC strains from ill children in our field site, and nearly all of the strains produced ST only. This serotype may be peculiar to the ETEC strains found in this setting, as it has not been associated with ETEC either from adult travelers to Egypt (42, 43) or from children or adults in other geographic regions. Since the most common serotypes observed in our setting are ones that do not generally predominate in other regions and since there is limited evidence that ETEC O and H antigens are protective (22, 28, 31), there would seem to be little to gain from incorporation of specific O or H antigens into a vaccine against ETEC.

The wide array of phenotypic combinations observed in these ETEC strains and the relative paucity of CF-positive strains limited our ability to discern associations among toxin profiles, adhesin type, and serotypes. A few observations, however, were noteworthy. While expression of CFA/II has been associated with LT and ST production in many studies (4, 6, 9, 15, 16, 18, 19, 35, 40), we found that all CFA/II-positive ETEC strains produced ST only. PCFO159 is most often found in association with its namesake, serotype O159:H4, and in one study, as many as 60% of selected O159:H4 ETEC strains expressed this putative adhesin (37). By contrast, in the present study none of 13 serogroup O159 ETEC strains, all but one of which were also serogroup H4, expressed PCFO159.

The basis for the great serotypic diversity and relative laxity of restrictions on CF type, toxin profile, and serotype combinations seen in this population-based collection of ETEC is open to debate. It has been proposed that the nonrandom associations between specific toxin profiles, adhesin types, and serotypes is a function of the differential stability of virulence plasmids in various *E. coli* groups (42). Since much of the world database on ETEC phenotypes has originated from hospital or clinic-based studies (42), it may be that this sampling strategy is biased toward selection of those strains that are either inherently more stable, more pathogenic, or both. We suspect that it was the sampling method in our study, namely, selection of all ETEC strains associated with community-acquired diarrhea in a defined location and time period, that led to selection of a greater diversity of ETEC strains. This, in turn, may be a reflection of the fact that there is a larger pool of environmental isolates out of which more stable combinations arise and persist over place and time, while those that are less stable or less pathogenic are more transient.

Current strategies for development of a vaccine against ETEC have focused on LT, CFA/I CFA/II, and CFA/IV as candidate antigens given their wide distribution and demonstrable protective effect (2, 12, 33, 42). Of the diarrhea-associated strains from this pediatric population, only 18% expressed one of these CF types. This contrasts markedly with findings from a survey of strains from U.S. military forces deployed to Egypt or Saudi Arabia, among which 75% of diarrhea-associated ETEC strains expressed CFA/I, CFA/II, or CFA/IV (43). Surveys in other geographic regions have found substantial variations in the proportion of diarrhea-associated ETEC strains that express these common CFs, ranging from 22 to 77% (6, 11, 15, 26, 29). Importantly, 44% of the ETEC strains from Egyptian children produced ST only and did not express CFA/I, CFA/II, or CFA/IV and thus would not be expected to be covered by a vaccine based on anti-LT, anti-CF immunity. It is possible that the low CF detection rate that we observed was in part artifactual, due to the loss of CF plasmids during processing or to the lack of CF expression. For comparison, in a recently completed, similarly designed community-based study performed in a nearby village area, we have found that 59% of ETEC isolates from children with diarrhea failed to express any one of the same battery of CFs tested in the present study (41).

Our findings emphasize the continued need to identify additional protective antigens commonly expressed by ETEC for inclusion in future vaccines, such that the range of pathogens covered by the vaccines may be broadened. The most logical target would seem to be ST, although attempts to design a sufficiently immunogenic ST-conjugate antigen that confers protection against toxin-homologous ETEC strains have met with limited success (14, 20, 25). Identification of other prevalent CFs may suggest additional target antigens for inclusion in a vaccine. While our tests for CS7, CS17, PCFO166, PCFO159, and CFA/III turned up few additional CF-positive strains, it remains possible that other adhesins for which we either did not assay or which are as yet undiscovered are prevalent in this region.

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