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# Typical Enteroaggregative and Atypical Enteropathogenic Types of *Escherichia coli* Are the Most Prevalent Diarrhea-Associated Pathotypes among Brazilian Children▽

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**A 1-year prospective study was carried out in two large urban centers of São Paulo State, Brazil, to determine the prevalences and roles of the different *Escherichia coli* pathotypes in children less than 5 years of age with diarrhea presenting to the emergency rooms of public hospitals or visiting private pediatricians' offices. Of the pathotypes sought, typical enteroaggregative and atypical enteropathogenic types of *E. coli* were isolated for 8.9% and 5.4% of 774 diarrhea cases, respectively, and were found to be dominant and significantly associated with diarrhea.**

Diarrheagenic *Escherichia coli* strains are major pathogens associated with enteric disease worldwide. *E. coli* strains are among the most important bacterial causes of childhood diarrhea. As many as six categories of *E. coli* that differ in their virulence factors have been described to date: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC) or Shiga toxin-producing *E. coli* (STEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (12).

The most commonly reported diarrheagenic *E. coli* organisms in Brazil are EPEC strains (3, 6, 10, 17, 21). In the city of São Paulo, EPEC has been considered the main cause of childhood diarrhea and in some studies has been found in up to 30% of children of low socioeconomic level (6, 21) (Table 1).

For many years, diagnosis of EPEC was based on O:H serotype identification. During the last 2 decades, the pathogenic mechanism of EPEC infection has been clarified, resulting in a change in diagnostic methods from serogrouping to phenotypic and genotypic methods. EPEC is now classified into two subcategories on the basis of hybridization with a probe to the EAF virulence plasmid: EPEC strains that hybridize with the EAF probe are designated "typical EPEC" (tEPEC), whereas EPEC strains that do not hybridize with the EAF probe are designated "atypical EPEC" (aEPEC) (12). While tEPEC strains are important pathogens, the importance of aEPEC is unknown.

In recent epidemiological studies conducted in Brazil, aEPEC strains have outnumbered tEPEC strains as causative agents of diarrhea in children (1, 5, 7, 14). This finding coincides with a decline in the number of diarrhea cases in several regions of Brazil. In developed countries, aEPEC strains have become a more frequent cause of diarrhea than tEPEC, and the same shift may be occurring in Brazil (22).

To better evaluate the extent of the involvement of aEPEC strains with diarrhea and to determine whether aEPEC strains are emerging pathotypes that are replacing the previously common tEPEC strains, we undertook a 1-year prospective study in two large cities of São Paulo State, Ribeirão Preto (population, >2 million) and São Paulo (population, >10 million). The purpose of the study was to determine the relative prevalences and roles of the different *E. coli* pathotypes in children with diarrhea presenting to the emergency rooms of public hospitals (PH) or visiting private pediatricians' offices (PO) outside the public health system. The first cohort represented children from low-socioeconomic-level families (average income, U.S. $180 per month), a group highly susceptible to EPEC-related diarrhea (3, 6, 10, 17). The second cohort represented a rarely studied group of children of the highest socioeconomic level (average income, U.S. $1,500 per month), who are able to afford the cost of a private physician (consulting fee, U.S. $100).

The first patient group consisted of 446 poor urban children up to 5 years old with diarrhea presenting to the emergency room of PH in the city of Ribeirão Preto (April 2002 to September 2003), which provides free medical assistance to urban children from low-socioeconomic-level families. The second patient group consisted of 328 urban children less than 5 years of age with diarrhea visiting private PO outside the PH system in the city of São Paulo (August 2002 to September 2003). The PO group was identified through personal contacts by study investigators. PO stool samples were sent to a high-standard reference private diagnostic center (Laboratory Fleury Medicina Diagnóstica, São Paulo, SP, Brazil). A mixed control population of 139 healthy children less than 5 years old was randomly selected irrespective of socioeconomic status from day care centers. This study was approved by the Universidade Federal de São Paulo, Escola Paulista de Medicina, Ethical Committee for human experimentation.

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TABLE 1. Prevalences of childhood diarrhea-associated EPEC strains in different urban centers of Brazil (1983 to 1995)

| Site (reference) | Yr | Children's age | Rate of EPEC isolation (%) | |
|---|---|---|---|---|
| | | | Cases | Controls |
| Brasília (3) | 1995 | <2 yr | 42.0 | 22.5 |
| Rio de Janeiro (17) | 1990 | <2 yr | 32.7 | 14.0 |
| Fortaleza (8) | 1983 | <1 yr | 4.6 | 3.1 |
| São Paulo (21) | 1983 | <6 mo | 38.0 | 7.0 |
| São Paulo (6) | 1991 | <1 yr | 30.0 | 4.6 |

All stool samples collected from patients and controls were placed in Cary-Blair transport medium, transported to a microbiology laboratory, and inoculated on primary media within 24 h. The samples were then plated on media selective for enteric pathogens and inoculated into enrichment broths, which were subcultured after overnight incubation at 37°C. For most bacterial enteropathogens, MacConkey agar was used. For EIEC, *Shigella* and *Salmonella* species, and *Yersinia enterocolitica*, *Salmonella-Shigella* agar was also used. For *Salmonella* species, tetrathionate enrichment broth with 40 μg/ml of novobiocin was inoculated and later subcultured onto brilliant green agar. *Campylobacter* species were detected on modified Skirrow plates under microaerophilic conditions (2).

For each child, four lactose-fermenting isolates and two non-lactose-fermenting colonies selected from MacConkey agar were stored on tryptic soy agar and sent to the research laboratory at Universidade Federal de São Paulo, São Paulo, Brazil. The strains were cultivated in commercial test systems (PROBAC, São Paulo, Brazil) for biochemical confirmation of species or genus. *E. coli* strains were typed using slide agglutination with polyvalent and monovalent antisera (PROBAC, São Paulo, Brazil) against the following O antigens of EPEC serogroups and EHEC: O26, O55, O86, O111, O114, O119, O125, O126, O127, O128ab, O142, O157, and O158. EPEC and EHEC strains that did not react with these O antisera were serotyped at the Instituto Adolfo Lutz (São Paulo, Brazil) using antisera O1 to O173 and H1 to H56.

All *E. coli* isolates were tested with specific DNA probes designed to detect EPEC (*eae* and EAF probes), EAEC (AA probe), DAEC (*daaC* probe), ETEC (heat-labile enterotoxin [LT] and heat-stable enterotoxin [ST] probes), EIEC (Inv probe), and EHEC/STEC (*Ehly*, $stx_1$, and $stx_2$ probes) (12). The probes were labeled with [α-$^{32}$P]dCTP, and colony hybridization assays were performed as described previously (18). The presence of the genes encoding the transcriptional activator AggR (*aggR*), aggregative-adherence AA fimbriae (AAF/I) (*aggA*) and AAF/II (*aafA*), plasmid-encoded toxin (*pet*), enteroaggregative heat-stable enterotoxin EAST1 (*astA*), and *Shigella* enterotoxin 1 (*shET1*) were tested by PCR as described previously (25).

Data for patients and controls were compared by a two-tailed chi-square or Fisher's exact test.

In total, 913 fecal samples were examined for enteric bacterial pathogens, 774 from children with diarrhea and 139 from children without diarrhea (Table 2). A total of 247 diarrheagenic *E. coli* strains were isolated from the 913 fecal specimens: 224 were identified as the only bacterial pathogen in the stools of children with diarrhea (111 from the PH group and 113 from the PO group), 17 were from controls, and 6 were found with another bacterial pathogen. Excluding *E. coli*, bacterial pathogens were isolated from less than 2% of diarrhea patients. *E. coli* isolates were tested with DNA probes to classify them into the different pathotypes.

TABLE 2. Prevalences of bacterial pathogens in children with diarrhea <5 years old attending PH or presenting to PO and healthy children (controls)

| Pathogen | No. (%) of children | | |
|---|---|---|---|
| | PH ($n$ = 446) | PO ($n$ = 328) | Controls ($n$ = 139) |
| *E. coli* pathotypes | | | |
| EPEC (total) | 25 (5.6) | 19 (5.8) | 2 (1.4) |
| Typical (*eae* EAF$^+$) | 2 (0.4) | 0 | 1 (0.7) |
| Atypical (*eae* EAF$^-$) | 23 (5.1)[a] | 19 (5.8)[a] | 1 (0.7) |
| ETEC (total) | 4 (0.9) | 5 (1.5) | 0 |
| LT only | 3 (0.7) | 3 (0.9) | |
| ST only | 1 (0.2) | 1 (0.3) | |
| LT and ST | 0 | 1 (0.3) | |
| EIEC | 2 (0.4) | 2 (0.6) | 1 (0.7) |
| EHEC (*eae* Stx1$^+$) | 2 (0.4) | 2 (0.6) | 0 |
| STEC (total) | 3 (0.7) | 0 | 0 |
| Stx1 | 2 (0.4) | 0 | |
| Stx1and Stx2 | 1 (0.2) | 0 | |
| EAEC | 33 (7.4)[a] | 36 (11.0)[a] | 2 (1.4) |
| DAEC | 42 (9.4) | 49 (14.9) | 12 (8.6) |
| Non-*E. coli* pathotypes | | | |
| *Campylobacter* spp. | 0 | 3 (0.9) | 0 |
| *Salmonella* spp. | 5 (1.1) | 0 | 0 |
| *Shigella* spp. | 5 (1.1) | 0 | 0 |
| Combination of pathogens | | | |
| EAEC + *Campylobacter* spp. | 0 | 2 (0.6) | 0 |
| EAEC + *Shigella* spp. | 2 (0.4) | 1 (0.3) | 0 |
| ETEC + *Salmonella* spp. | 0 | 1 (0.3) | 0 |

[a] $P$ value <0.001 versus control.

The bacterial isolation rate in the PO group was greater than in the PH group: 36.6% [120 of 328] compared with 27.6% [123 of 446], respectively. Interpretation of this discrepancy is difficult, since some enteric pathogens, such as virus and parasites, were not screened. In a recent survey carried out to investigate the etiology of childhood diarrhea in the city of Botucatu, another urban center of São Paulo State, rotavirus was detected in 15% of diarrheic children attending a public health center (15). Thus, rotavirus could be the diarrheal agent in those children in whom no bacterial pathogen was identified.

Of the six recognized pathotypes of *E. coli*, all but DAEC were recovered from children with diarrhea and were also recovered from children without diarrhea, although much less frequently.

EAEC was isolated significantly more often from both diarrheal groups than from healthy controls ($P < 0.001$). The rate of isolation for EAEC in the PO group (11% [36 of 328]) was slightly higher than in the PH group (7.4% [33 of 446]). All EAEC isolates were tested by PCR to detect genes for the proposed EAEC virulence factors, such as AggR, AAF/I, AAF/II, Pet, EAST1, and *Shigella* enterotoxin 1. The AggR regulon was present in all strains. Several different combinations of the virulence markers were found among the EAEC

TABLE 3. Prevalences of virulence-related markers among EAEC strains isolated from children with diarrhea <5 years old attending PH or presenting to PO and healthy children (controls)

| Genetic profile | No. (%) of children | | | |
|---|---|---|---|---|
| | Total ($n$ = 71) | PH ($n$ = 33) | PO ($n$ = 36) | Controls ($n$ = 2) |
| *aggR aafA astA pet shET*1 | 4 (5.6) | 2 (6.0) | 2 (5.6) | 0 |
| *aggR aggA astA pet* | 2 (2.8) | 2 (6.0) | 0 | 0 |
| *aggR aggA astA shET*1 | 4 (5.6) | 2 (6.0) | 2 (5.6) | 0 |
| *aggR aafA astA pet* | 1 (1.4) | 0 | 1 (2.8) | 0 |
| *aggR astA pet shET*1 | 1 (1.4) | 1 (3.0) | 0 | 0 |
| *aggR aggA astA* | 2 (2.8) | 1 (3.0) | 1 (2.8) | 0 |
| *aggR astA shET*1 | 5 (7.0) | 1 (3.0) | 4 (11.1) | 0 |
| *aggR pet shET*1 | 2 (2.8) | 0 | 2 (5.6) | 0 |
| *aggR aggA* | 3 (4.2) | 0 | 3 (8.3) | 0 |
| *aggR astA* | 5 (7.0) | 4 (12.1) | 1 (2.8) | 0 |
| *aggR pet* | 1 (1.4) | 0 | 1 (2.8) | 0 |
| *aggR shET*1 | 27 (38.0) | 14 (42.4) | 13 (36.1) | 0 |
| *aggR* | 12 (16.9) | 6 (18.2) | 6 (16.7) | 2 (100) |

isolates (Table 3). The most prevalent combination was *aggR* and *shET1*, found in 14 (42.4%) and 13 (36.1%) strains from the PH and PO groups, respectively.

aEPEC was the second most prevalent diarrhea-associated pathotype in the two groups of children studied, while tEPEC was rarely detected. aEPEC strains occurred at similar frequencies in both diarrheal groups: 5.1% [23 of 446] in the PH group compared with 5.8% [19 of 328] in the PO group. The serogroups of these aEPEC isolates are indicated in Table 4. Of the 23 strains from the PH group, 15 (65%) belonged to recognized EPEC serogroups (O26, O55, O119, O127, O128, and O142). Among the 19 strains from the PO group, 16 (84%) either belonged to non-EPEC serogroups or did not react with antisera O1 to O175. One strain from the control group belonged to the O125 serogroup. A total of 22 (51.2%) aEPEC strains were *astA* positive: 12 (52.2%) from the PH group and 10 (52.6%) from the PO group. Only two tEPEC strains were isolated in this study, both from the PH group. One was an O142 strain, and the other was an O127 strain. One O125 tEPEC was recovered from the control group.

In addition, we identified nine ETEC (1.2%), four EHEC (0.5%), and three STEC (0.7%) strains in children with diarrhea. Most of the ETEC strains isolated were LT positive. Almost all the EHEC and STEC isolates were $stx_1$ positive. Three EHEC isolates belonged to the O103:H2 serotype and were analyzed for genetic relatedness by pulsed-field gel electrophoresis using standard methods (20). Pulsed-field gel electrophoresis analysis of SpeI-digested genomic DNA revealed different genotypes, suggesting that these isolates are not clonal (data not shown). EIEC strains were recovered from both diarrheal (0.5%) and control (0.7%) groups.

This study indicates that EAEC and aEPEC may play significant roles as agents of childhood diarrhea in Brazil. EAEC was by far the most prevalent diarrhea-associated pathotype in each of the two groups of children studied and accounted for 8.9% of the total of 774 diarrhea cases. This is the second case-control study that we have conducted in the city of São Paulo (19). In the first study, EAEC was also found to be dominant and significantly associated with infantile diarrhea. As demonstrated in this and previous work, EAEC appears to

TABLE 4. Serotypes of aEPEC isolates from children with diarrhea <5 years old attending PH or presenting to PO and healthy children (controls)

| Group of children | Serotype(s)[a] (no. of isolates) |
|---|---|
| PH ($n$ = 23) | O4:HNT (1), O26:HNT (2), O55:HNT (2), O119:HNT (3), O127:NM (2), O128:NM (1), O142:HNT (5), O157:HNT (1), ONT:HNT (5), OR:NM (1) |
| PO ($n$ = 19) | O9:HNT (2), O33:HNT (2), O35:H19 (1), O55:HNT (1), O119:HNT (2), O152:HNT (1), O153:H2 (1), O167:H6 (1), ONT:H9 (1), ONT:H10 (1), ONT:H12 (1), ONT:H19 (1), ONT:H21 (2), ONT:NM (1), OR:NM (1) |
| Controls ($n$ = 1) | (O125:HNT) |

[a] NT, nontypeable; NM, nonmotile; R, rough strain.

have become a major etiologic agent of diarrhea in São Paulo. Several other studies conducted in Brazil have also shown that EAEC strains are frequently detected in children with diarrhea (4, 13, 14, 15, 16, 25). Taken together, these findings suggest that EAEC is emerging as a significant enteric pathogen, responsible for acute and persistent diarrhea in Brazilian children.

Epidemiologic and pathogenetic studies have suggested that AA probe-positive strains, which are predicted to carry the AggR regulon, may be the true EAEC pathogens (11). The term "tEAEC" has emerged to denote such EAEC strains. Based on this classification, our EAEC isolates were renamed tEAEC. As is typical for EAEC, the strains identified in this study were heterogeneous with respect to virulence gene content (13, 25).

Although recent studies conducted in Brazil have shown that aEPEC is more frequently detected in children with diarrhea, no epidemiological association with diarrhea has been found in Brazil until now. In our study a strong association of aEPEC with diarrhea was found, in accordance with many studies in developed countries (12, 22). This finding reinforces the necessity for further investigation of the virulence properties of aEPEC strains. The analysis of the presence of the *astA* gene, which has been suggested to contribute to the virulence of this pathotype (1, 24), showed that half of the isolates harbored the gene. Serotyping of the aEPEC strains revealed two kinds of atypical strains: strains that belong to recognized EPEC serogroups, like tEPEC, and strains that do not belong to EPEC serogroups that may resemble EHEC, EAEC, or DAEC (22). Interestingly, children from the PH group were most frequently colonized with the EPEC serogroups, whereas children from the PO group were infected with strains of non-EPEC serogroups. Several atypical strains of non-EPEC serogroups have also been identified in other epidemiological studies of diarrhea in children admitted to PH, some of which were isolated in the city of Ribeirão Preto (1, 7, 14).

The very low rate of tEPEC in our study was somewhat surprising but is consistent with a trend that has been recently observed in Brazil. Until the 1990s, tEPEC strains were the main cause of infantile diarrhea in Brazil (3, 6, 10, 21), but it seems that they are becoming more and more rare. The reason for this decrease in the tEPEC frequency is not known, but it may be due to improvements in public health measures, such

as sanitary conditions. Indeed, the few tEPEC strains found were recovered from the PH group, a group of patients more susceptible to EPEC-related diarrhea in the past.

The detection of a few EHEC strains in both diarrhea groups was not surprising. Non-O157 EHEC strains have been circulating as agents of infantile diarrhea in the state of São Paulo since the late 1970s, with serotypes O111:NM, O111:H8, O26:H11, and O103:H2 accounting for most of the cases (9, 23). The continuing presence of these strains in São Paulo is certainly a public health concern. Regarding EHEC and both categories of EPEC, the present situation in Brazil is becoming similar to the one that exists in developed countries, where tEPEC strains are very rare and aEPEC and EHEC are relatively frequent.

In conclusion, this study suggests that EAEC and aEPEC may have become major etiologic agents of childhood diarrhea in Brazil. Further studies are needed to investigate the pathogenic mechanisms of these *E. coli* pathotypes, which will allow development of better diagnostic tests.

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