Prevalence of enteropathogens in normal feces from healthy children at an infant day care in Brazil

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Abstract
Introduction: The diarrhea associated with gastroenteritis is a major cause of morbidity and mortality worldwide, affecting mainly infants. The characterization of both viral and bacterial agents associated with gastroenteritis can establish policies for surveillance, prevention and treatment of infections. Group A rotaviruses are the major infectious agent associated with dehydration in children, followed by pathotypes of *Escherichia coli*. There are three main types of clinical infections caused by *E. coli* strains that have acquired virulence genes: (i) enteric and diarrheal diseases, (ii) urinary tract infections, and (iii) sepsis and meningitis.

Methodology: In this study, the objective was to identify the presence of rotavirus and diarrheogenic *E. coli* in the feces of children 4 to 14 months of age who displayed no gastroenteritis symptoms and stayed all day in a day-care center. We analyzed 188 samples using PAGE and PCR to identify rotaviruses and *E. coli* virulence genes, respectively.

Results: Thirty-six samples (19.1%) were positive for at least one pathotype of *E. coli*. Nineteen were identified to be of the EPEC group and fifteen of the EAEC group. Rotaviruses were not identified.

Conclusions: As EPEC and EAEC are potential pathogens for children less than one year of age or immunocompromised individuals, our results show the importance of appropriate monitoring by public health agencies. In the situation that we have studied, children can be considered asymptomatic carriers of these pathogens and can transmit them to other susceptible children.

Key words: enteropathogen; children; day-care center; *Escherichia coli*; rotavirus


(Received 24 March 2011 – Accepted 14 August 2011)

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Introduction
Diarrhea is the major cause of morbidity and mortality worldwide, mainly affecting children less than five years of age. In the world, the probability of transmission of infectious agents among children in a nursery school ranges from 50% to 71% during outbreaks. Moreover, these rates are lower (15% on average) in children not exposed to that environment [1]. In developing countries, viruses, bacteria, and parasites are associated with outbreaks of gastroenteritis in day-care centers. Among them, rotavirus and diarrheogenic *Escherichia coli* (DEC) are considered to be the most common enteropathogenic organisms [2].

In general, rotaviruses are responsible for 20% to 60% of cases of hospitalization due to diarrhea [1]. The infection frequently occurs in the winter in temperate climates, but in tropical settings and in developing countries a defined seasonality has not been observed [3]. In Brazil, rotavirus cases are mainly observed in settings with high human contact, such as schools, day-care centers, hospitals and big families [4]. Therefore in 2006, immunization of infants with two doses of RV1 vaccine was initiated [5]. Since then, cases of rotavirus disease in children have decreased significantly while other enteropathogens are incriminated in diarrheic outbreaks in children. Studies in the city of São Paulo, Brazil, showed predominance of diarrheic *Escherichia coli*, mainly enteropathogenic *E. coli* (EPEC), in 40% of cases of acute diarrhoea [6].

Based on symptoms and specific virulence factors, diarrheic *E. coli* are classified into six categories: enteropathogenic *E. coli* (EPEC), enterohaemagglutigative *E. coli* (EAEC), shigatoxigenic *E. coli* (STEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC) and enteroinvasive *E. coli* (EIEC) [7].
EPEC are associated with infantile diarrhea. The principal protein ( intimin) involved in this phenotype is codified by the eae gene. Typical strains of EPEC harbor the eae gene and the EAF plasmid (EPEC adherence factor plasmid). Atypical strains have eae gene but lack the EAF-plasmid, while other atypical EPEC contain parts of the plasmid but do not express bundle-forming pili for adherence [8]. STEC are the E. coli strains that only produce shiga toxin (STx) whereas EHEC produce Stx and have the capability to induce A/E lesions [7].

ETEC has been associated with watery diarrhea in children and traveller’s diarrhea. This group is distinguished by the ability to produce enterotoxins, heat stable enterotoxins (STs), codified by estl gene and/or heat labile toxins (LTs), expressed by eltI gene [7].

EAEC are usually distinguished by an aggregative adherence pattern on HEp-2 cultured cells. Certain strains carry a high-molecular-weight plasmid (pAA) associated with aggregative adherence [9] on which a number of virulence genes are located, including an antiaggregation protein transporter gene (aat; previously referred to as CVD432) [10] and an enteroaggregative heat stable toxin (EAST) gene (astA). EIEC group has the capacity to invade tissue, causing dysentery [7].

EIEC are classified biochemically as E. coli but share many properties, including virulence mechanisms, with Shigella. The organisms possess a large invasion plasmid, which codes the Mxi-Spa type III secretion system and invasion plasmid antigen (Ipa) effectors, conferring upon these bacteria the ability to invade eukaryotic cells. The ipaH gene can be used as molecular marker for PCR [8].

In spite of the availability of modern, rapid, and easy-to-use diagnostic techniques, such as virulence gene-specific PCR, screening for DEC categories is not routinely performed in Brazilian clinical laboratories. Thus the objective of this study was to screen rotavirus-immunized children for the presence of different pathotypes of E. coli. We also aimed to verify whether children could have asymptomatic infections with diarrheogenic pathogens and function as carriers in the population.

Methodology

Study population

From May (autumn) to August (winter) of 2006, a total of 94 children who stay all day in an infant day-care center in Unicamp, Brazil, were screened for the presence of viral and bacterial enteric agents and were monitored throughout the period for signs of diarrhea. The staff feed the children meals that are prepared at the day care 3 times per day. The children could also be breast-fed by their mothers twice per day. All children received rotavirus vaccine (Rotarix, Glaxo Smith Kline Biologicals, Middlesex, United Kingdom) administered by the Brazilian public health system.

Sampling

Two stool samples were collected for each child and a total of 188 set samples of feces were analyzed. All children were monitored throughout the period for signs of diarrhea. This study was approved by the Ethics Committee for Research on Humans of the State University of Campinas (UNICAMP), with ID number 483/2004.

Identification of rotavirus by PAGE

The samples were screened by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (7.5%) after phenol-chloroform extraction of viral double-stranded RNA. Polyacrylamide gels were silver stained [11].

Bacterial identification and detection of virulence factors of diarrheic E. coli

Fecal samples were plated onto MacConkey agar (Difco) medium. A loopful of bacterial growth or ten E. coli-like colonies taken from the first streaking area of the fecal culture plates was suspended in 0.5 mL of sterile water and boiled for five minutes to release the DNA which was subsequently subjected to PCR. If no positive colony was found among the first ten, at least 40 more colonies were tested. All the E. coli isolates were subsequently characterized using biochemical identification [12,13].

Pathotypes of E. coli were detected by PCR using specific primers for the following seven E. coli virulence genes: stx1/stx2 in STEC and EHEC; eltI and estl in ETEC; eae in EPEC and EHEC; ipaH in EIEC and pCVD432 plasmid in EAEC (Table 1).

The PCR reactions were performed using 1X PCR Buffer; 2 mM MgCl2; 0.2 mM dNTP; 1.5 U Taq DNA polymerase (Fermentas, Burlington/Canada); 7 µL DNA template; and 60 ng of each primer. The products were analyzed by electrophoresis on a 1.5% agarose gel. The gel was stained with ethidium bromide and imaged under UV transillumination (LKB MacroVue Pharmacia, GE Life Science, Germany).
The identification of different diarrheogenic *E. coli* groups was assessed by means of the Chi-square test (Fisher’s exact test). Statistical significance was set at $p < 0.05$.

**Results**

In the 188 stool samples analyzed, 36 (19.1%) were PCR positive for at least one of the virulence factors associated with diarrheic *E. coli* (Table 2). Nineteen strains were positive for *eae* (EPEC) ($p > 0.05$); 15 strains were positive for pCVD432 (EAEC) ($p > 0.05$); one strain was positive for *stx1/stx2* (STEC) ($p < 0.05$); and one strain was positive for *eltI* (ETEC) ($p < 0.05$). One sample was positive for both EPEC and EAEC (Table 2). Rotaviruses were not identified in this study. In the infantile population studied, diarrheic *E. coli* levels were not significantly high ($p > 0.05$); however, the predominance of EPEC and EAEC in isolates from children is statistically significant.

**Discussion**

In developing countries, bacterial agents are the main cause of diarrhea in children whereas viral agents are more prominent in industrialized countries [14]. In day-care centres, the transmission of infection due to enteric pathogens and rotavirus is usually common among children with no evident signs and symptoms of gastroenteritis. However, many of these children may also have severe dehydration or be the source of exposure to their families [1]. Therefore, they act as silent carriers of infection and play an important role in the transmission of disease throughout the community.

It is important to check the presence of such pathogens in asymptomatic patients. In the present study, we analyzed 188 stool samples of children who attended a nursery in Brazil in a six-month period that included autumn and winter to verify if these children could be asymptomatic but still be rotavirus and *E. coli* carriers. Surprisingly, none of

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**Table 1.** Primers used on PCR methods for diarrheogenic *Escherichia coli* detection

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5’-3’)</th>
<th>T°C/Product (bp)</th>
<th>Reference</th>
</tr>
</thead>
</table>
| stx1 | F- AAGTTGCAGCTCTCTTTGAATA  
R- TGCAAACAAATTATCCCCCTGAG | 50/364 | 20 |
| stx2 | F- GGCGAGTTATTTTTGCTTGGA  
R- GTATCTGCTAAGCGGTAA | 50/386 | 20 |
| elt I | F- AGATATAATGATGGATATGTATC  
R- TAAACCTCGAAATAAAATCTC | 48/300 | 21 |
| ipaH | F- GGTCCGTAGGGCTTTCCCAGCCGGTAC | 50/600 | 22 |
| Eae | R- GGCGAGCTACCTCCTGAGAGTAC | 50/384 | 23 |
| pCVD432 | F- GGCGGAAAGACTGTATCAT  
R- CAATGGATAGAAATCCGCTTTT | 48/630 | 24 |
| est I | F- ATTTTATTTTCGTAATGCTTTT  
R- GGATTACAACACAGCTACAGCAGT | 48/176 | 25 |

*a: anneling temperature*

**Table 2.** Frequency of pathotypes of *Escherichia coli* isolated from children at Campinas, Brazil (2006)

<table>
<thead>
<tr>
<th>Diarrheic <em>E. coli</em></th>
<th>Autumn</th>
<th>Winter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fist set samples (n=70)</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Second set samples (n=34)</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Third set samples (n=50)</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Fourth set samples (n=34)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Number of positive samples (n=188) (%)</td>
<td>15 (7.9)</td>
<td>19 (10.1)</td>
</tr>
</tbody>
</table>

*The strains characterized were positive in the PCR methods for the genes studied. EAEC: positive for pCVD432 plasmid gene; EPEC: positive for *eae* gene; ETEC: positive for *eltI* gene; STEC: positive for *stx* gene.*
the samples was positive for rotavirus; however, 19.1% carried different pathotypes of diarrheic *E. coli*. EPEC was the most frequent category of diarrheic *E. coli* identified in children. Historically, EPEC has been considered a group that presents serotypes associated with infantile diarrhea [15]. This group can present both typical and atypical pathotypes. Atypical EPEC are strains with a diversity of genetic combinations involved with pathogenicity [7]. The role played by these EAF+ and EAF- strains outside the EPEC serogroups in endemic diarrhea has not been established. In industrialized countries, they have become a more frequent cause of diarrhea than typical EPEC, and the same shift may be occurring in Brazil [16]. Brazilian studies have shown identification of this group in samples of healthy and symptomatic children [14,15] and EPEC are more commonly isolated in healthy children than from children with diarrhea [15]. In this study, we detected EPEC in the feces of healthy children who stay all day in a day-care center. This observation indicates that apparently healthy children can be an important source of infection.

Another group of diarrheic *E. coli* identified in this study was enteroaggregative *E. coli* (EAEC). The majority of studies involving the detection of EAEC have been based on a hybridization test using target sequences of adhesion genes. Although the sensitivity of this test has varied in population studies, it is very specific [17]. Recently, PCR assays that target genes located on the pAA, including *aat*, *aap* and *aggR*, have been described. However, EAEC are a heterogeneous group, and not all strains that adhere to HEp-2 cells in a stacked-brick formation harbor the pAA plasmid. Studies have shown that the *aaiA* and *astA* genes are potential targets for detecting both groups [18]. EAEC are detected in 7.9% of the cases, surpassed only by the EPEC group (10.1%). Other studies in Brazil show that this category of diarrheic *E. coli* is frequently isolated from children with or without diarrhea [15].

Enterotoxigenic *E. coli* (ETEC) was detected in only one strain (0.5 %) in our study and these findings are not in agreement with those of another study in our country [17]; however, it is important to note that we analyzed healthy children. In this study, the ETEC detection rates were significantly lower than rates identified in other studies [14,15,17]. Enteroinvasive *E. coli* (EIEC) was also identified in only one strain. Cases of diarrhea by EIEC are endemic and sporadic [7]. In Brazil, EIEC could be isolated in children over two years of age when they present acute diarrhea [15]. In addition to the results mentioned above, we detected only one STEC strain, confirming previously published results which showed that STEC is not frequently identified in children with symptoms, but mainly in apparently healthy children [15].

In conclusion, these results show the potential of investigating endemic enteric pathogens in the community, because healthy individuals may have these pathogens as a member of their microbiota [14,18,19]. The occurrence of diarrheic *E. coli* in this population and possibly in children without subclinical rotavirus infection indicates that apparently healthy individuals can be sources of potential pathogens for diarrhea infection, representing a significant public health problem. These facts justify the development and implementation of rapid identification methods.

**Acknowledgements**

We are extremely grateful to Ana Silvia de Souza Lima Michelone, the nurse of the day-care center who supervised and organized the collection of stool samples. Also, we would like to thank to all the nurseries that helped with sample the collection, and the parents and children who participated in the study.

**References**


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Conflict of interests: No conflict of interests is declared.