Diarrheagenic *Escherichia coli* in acute gastroenteritis in infants in North-West Italy

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INTRODUCTION

Diarrheal disease is still a health problem, especially in developing countries, where it is considered one of the foremost causes of death in children, accounting for approximately 2 million deaths each year worldwide (Kosek et al., 2003; Bryce et al., 2005). Further, acute gastroenteritis could affect subjects of any age and status and also represents an important cause of hospitalization and morbidity in developed countries (Caprioli et al., 1996). The features of acute diarrhea vary from place to place depending on local meteorology, geography, and socioeconomic variables. Knowledge of the major etiologic agents of this disease is important for epidemiological surveillance and correct treatment (Yamashiro et al., 1998). The different diarrhoeal syndromes can be caused by bacterial, viral and parasitic infections of ei-
ther single or multiple aetiology (Ochoa et al., 2004; Robins-Browne et al., 2004a; O’Ryan et al., 2005).

Diarrheagenic _E. coli_ pathotypes represent a leading bacterial cause of pediatric diarrhea in developing regions, with some responsible for traveler’s diarrhea, and are also an emerging cause of diarrhea in industrialized countries (Nataro & Kaper, 1998; Choen et al., 2005). _E. coli_ is usually found in the commensal intestinal bacterial flora, but it could become a pathogen through acquisition of genetic determinants, which may enhance adhesiveness or toxicity. These two factors make _E. coli_ particularly aggressive in infants (Nowrouzian et al., 2003). _E. coli_ strains associated with diarrhea have been classified into six groups, based on clinical, epidemiological and molecular criteria: enteropathogenic _E. coli_ (EPEC), enterohaemorrhagic _E. coli_ (EHEC), enteroinvasive _E. coli_ (EIEC), entero-toxigenic _E. coli_ (ETEC), enteroaggregative _E. coli_ (EAggEC) and diffusely adherent _E. coli_ (DAEC) (Nataro and Kaper, 1998; Guion et al., 2008).

Our interest was to investigate the prevalence of the enteropathogenic _E. coli_ in clinically relevant (i.e., hospitalization-requiring) infantile gastroenteritis, using highly sensitive molecular techniques as diagnostic tools. A further aim was to determine the distribution of co-infections between _E. coli_, bacterial and viral agents, and the correlation between gastrointestinal infection and infant age.

**METHODS**

**Subjects and sample collection**

Between March 2008 and June 2009, a total of 160 stool samples from infants admitted to the Department of Pediatric and Adolescence Science (Regina Margherita Children Hospital, Turin, Italy) with a diagnosis of acute gastroenteritis were submitted to the laboratory. Infants mean age was 272±148 days, they were 92 males and 68 females, born at term and appropriate for gestational age. Patients presented diarrhea, defined by stool volume higher than 10 ml/kg/die or liquid stools, with or without vomiting. Infants were excluded in case of vomiting but no diarrhoea, or if they suffered from concomitant illness, chronic or malformative diseases.

At enrolment, each infant underwent a clinical examination and parents were interviewed to obtain data on gestational age, kind of delivery (spontaneous or caesarean), birth weight, and type of feeding (exclusively or partial breast-feeding or formula-feeding). Informed consent was obtained from each child’s parent.

Stool samples were requested from enlisted patients within the first 48 hours of hospitalization. Children had not received any antibiotic therapy in the week preceding the sampling. About 5-10 g faeces were collected for each subject directly from the nappy. Each sample was stored at -80°C, immediately after collection, in a numbered screw-capped plastic container in aerobic conditions, until they were processed.

**Detection and isolation of _E. coli_ strains**

Faecal samples were collected from all infants. Coliform strains were isolated by homogenizing fresh samples (10% wt/vol) with sterile saline (0.9% NaCl). The homogenates were filtered through a 100 µm metal sieve and serially diluted in the same solution. One hundred microlitres of each dilution were seeded on the selective McConkey-sorbitol agar plates (VWR™ MERCK, Germany), utilizing quadrant streaking methods to produce isolated colonies, and incubated overnight at 37°C in 5% CO₂ atmosphere.

From each plate three different colonies, identified as _E. coli_ on the basis of their morphology were inoculated in 5 ml TSB and incubated overnight at 37°C. An aliquot of overnight bacterial culture was subjected to biochemical identification by API 20E system (Biomerieux, France). The isolated strains were also screened for possible haemolytic activity on Blood Agar plates containing 5% sheep erythrocytes washed three times with PBS (pH 7.2). The plates were incubated at 37°C in aerobic conditions and were examined for haemolytic activity.

DNA was extracted with the NucleoSpin Tissue method genomic DNA (Macherey-Nagel, USA) and was stored at -20°C until further use. For each isolate one µl of DNA obtained as described above, corresponding to about 5x10⁵ cells, was processed by each multiplex PCR assay for the presence of genes _lt_ and _st_ (Moseley et al., 1982; Sears & Kaper, 1996) (ETEC), of genes _eaeA_
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Table 1 - Primer sequences and predicted lengths of PCR products.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Oligonucleotide sequence (5’ To 3’)</th>
<th>Amplicon Size (Bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>elt</td>
<td>F:TCTCTATGTGCATACGGAGC R:CCATACTGATTGGCAGCAT</td>
<td>322</td>
<td>Orjan &amp; Strockbine (1993)</td>
</tr>
<tr>
<td>sta</td>
<td>F:TCTTCCCCTCCTTTATGTCAGTC R:CCAGCACAGGCAAGGATAC</td>
<td>170</td>
<td>Rappelli et al. (2001)</td>
</tr>
<tr>
<td>eaeA</td>
<td>F:TATAAGCTGAGTGCTGAAAT R:CTGAGATCGAGAGGATTAC</td>
<td>229</td>
<td>Calundungo et al. (1994)</td>
</tr>
<tr>
<td>bfpA</td>
<td>F:CACCGTACCAGCAGGATGTA R:GTGGCCGCTTCAGGAGGAT</td>
<td>450</td>
<td>Calundungo et al. (1994)</td>
</tr>
<tr>
<td>stx1</td>
<td>F:GAAGAGTCCGGAGGATTACG R:AGGATGCAAGATTCATTAAAT</td>
<td>130</td>
<td>Pollard et al. (1990)</td>
</tr>
<tr>
<td>stx2</td>
<td>F:GGGTAAGCTGGCATTACGGG R:GCTCGTAAGTAGAGTTCTG</td>
<td>510</td>
<td>Calundungo et al. (1994)</td>
</tr>
<tr>
<td>uidA</td>
<td>F:CAAAAGCCAGCAGGAT R:GCACAGACATCAAAGAG</td>
<td>623</td>
<td>McDaniels et al. (1996)</td>
</tr>
</tbody>
</table>

( Jerse et al., 1990) and bfpA (Donnenberg et al., 1992) (EPEC), and of genes stx1 and stx2 (O’Brien & Holmes, 1987) (EHEC). A primer pair specific for the uidA gene coding for β-glucuronidase (McDaniels et al., 1996) was also used for the confirmative species identification of E. coli. Details of the nucleotide sequence, the specific reference, and the predicted length of resulting amplicon for each primer pair are listed in Table 1.

PCR was performed in a 25 μl reaction mixture containing 12.5 μl PCR Master Mix (2X) (Fermentas, Canada), 1 μM (each) primer, 1 μl template DNA and water nuclease-free to 25 μl. The thermocycle program used for all amplifications consisted of the following time and temperature profile: 5 min at 94°C; 30 cycles of 1 min at 94°C, 1 min of annealing (ranging from 55°C to 58°C), 1 min at 72°C; 7 min at 72°C after the final cycle before cooling at 4°C.

The amplification products were electrophoresed through a 2% agarose gel and visualized with UV transilluminator after ethidium bromide staining. A 100 bp DNA ladder (Invitrogen, USA) was used as a molecular size marker in gel. Positive controls for PCR were E. coli ATCC 35401 (ETEC st+/lt+), E. coli ATCC 43887 (EPEC bfpA+/eaeA+) and E. coli ATCC 35150 (EHEC stx1+/stx2+/eaeA+); E. coli ATCC 25922 was used as negative control. The eventual cytotoxic activity and identification of any verocytotoxin strains were determined by Vero cell culture assay (Karmali et al., 1985).

Isolation of other enteric pathogens

All faecal samples were examined for Rotavirus and Adenovirus using enzyme immunoassay tests. Specimens were tested for Rotavirus antigen by using a commercial ELISA kit (Rotaclone, Meridian Diagnostics) (Coffin et al., 2006). Adenovirus was detected using commercial ELISA kit (Adenoclone, Meridian Diagnostics) (Rodriguez-Baez et al., 2002) with subsequent determination of serotypes 40 and 41 by Adenoclone 40, 41 (Meridian Diagnostics). All samples from hospitalized patients were examined for other enteric pathogens in addition to potential pathogenic E. coli, Rotavirus and Adenovirus, including Campylobacter spp, Salmonella spp., Shigella spp., using standard microbiology procedures (World Health Organization, 1987).

Statistical Analysis

In order to describe the characteristics of the studied sample, the main information was shown using classical descriptive indicators. Odds Ratio (OR) indicator and relative 95% confidence intervals (95% CI) were estimated [in the text showed in square brackets] to describe possible
different risks of pathogenic E. coli infections considering age (13-21 months versus 0-12 months).

RESULTS

E. coli were isolated in 75 of 160 collected samples (46.88%). Ten samples of the E. coli group (13.33%) had been positively recognised for genes related to pathogenesis: six were EPEC (two typical EPEC with the eae and bfpA genes and four atypical EPEC with only eae genes) (60% of pathogens), and four ETEC (one with the lt and st genes, one with only st gene and two with lt genes) (40% of pathogens).

All isolates were negative in Vero cell cytotoxicity test.

Others bacterial potentially pathogens were isolated in 60 samples (37.5% of the overall collected samples): 15 Salmonella spp., 8 Klebsiella pneumoniae, 9 Klebsiella oxytoca, 11 Citrobacter freundii, 5 Pseudomonas aeruginosa, 2 Serratia spp., 7 Enterobacter cloacae, 1 Shigella spp., and 2 Campylobacter spp.

Rotavirus was found in 35 samples (21.88% of the overall collected samples) and Adenovirus, serotypes 40 or 41, in 2 samples (1.3%).

In 8 of the 10 subjects with enteropathogenic E. coli infection, E. coli was the only potentially pathogenic microorganism observed, while in 2 of them there was a coinfection between E. coli and one or more infective agents such as viruses (Rotavirus). Therefore, data showed that the presence of diarrheagenic E. coli infection was 6.3%, but it becomes 5% considering E. coli as a unique agent responsible for diarrhea. The datum is not statistically meaningful, probably because of the small sample (p>0.05).

Table 2 shows the presence of pathogenic E. coli strains isolated on the basis of different age of patients. 3/106 infants aged 0-12 (2.8%) and 7/54 infants aged 13-21 months (12.9%) were positive for diarrheagenic E. coli [13-21 months versus 0-12 months OR=5.11, 95% CI from 1.12 to 26.28]. This is a significant difference even if the 95% CI shows a high variability of the estimated risk.

The seasonal prevalence of pathogenic E. coli had a strong reduction in autumn and winter (from October to January) and few seasonal peaks in March-May.

The data on Rotavirus and Adenovirus gastroenteritis showed both viruses presented a remarkable reduction in the summer months (from June to September).

DISCUSSION

The present study was performed to identify the incidence of E. coli as a potential aetiologic agent of diarrheal disease in a Children’s Hospital in North-West Italy. Previous reports have evaluated the prevalence of various non-Shiga toxin-producing E. coli as a cause of childhood diarrhea in the EU and in the US (Nataro & Kaper, 1998; Caeiro et al., 1999; Knutton et al., 2001; Choen et al., 2005).

Our study showed the importance of pathogenic E. coli gastroenteritis in clinical practice and in the surveillance of incidence and complications. This is possible by means of institutes such as the Istituto Superiore di Sanità in Italy, Enter-Net in the EU (Fischer, 1999) and Pulse-Net in the US. However, diarrheagenic E. coli strains are not routinely sought as stool pathogens in clinical laboratories.

<table>
<thead>
<tr>
<th>AGE (months)</th>
<th>Pathogenic E. coli</th>
<th>Not pathogenic E. coli</th>
<th>Not E. coli</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>13-21</td>
<td>7</td>
<td>28</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>0-12</td>
<td>3</td>
<td>37</td>
<td>48</td>
<td>18</td>
</tr>
</tbody>
</table>

OR*=5.11; 95% CI from 1.12 to 26.28

*OR was calculated comparing the pathogenic E. coli category with over all other categories.
We observed two categories of diarrheagenic *E. coli* associated with clinical illness: EPEC (typical and atypical) and ETEC. EPEC infection is primarily an illness concerning children less than 2 years of age (Nguyen et al., 2006). Atypical EPEC are now predominant in industrial countries, while typical EPEC are more frequently isolated in developing areas. Atypical EPEC constituted the *eaeA*-positive isolates. This finding is in accordance with reports from the UK (Knutton et al., 2001) and Brazil (Dulguer et al., 2003).

*E. coli* was the only potential pathogen isolated in 5% of the analyzed samples; although this result is not statistically significant due to the small number of positive samples, 5% of children were discharged without identification of the aetiologic agent responsible for gastroenteritis. This lack of association with the risk bound to the possible spread of the infection.

Rotavirus was the predominant single pathogenic etiologic agent identified (21.88%) (Rodriguez-Baez et al., 2002; Coffin et al., 2006; Forster et al., 2009), while routine bacterial enteropathogens (*Salmonella spp.*, *Shigella spp.*, *Campylobacter spp.*) were identified in 18 (11.25%) of studied subjects.

Rotavirus and diarrheagenic *E. coli* co-infection has been shown in some subjects and it could be considered a risk factor for more highly symptomatic illness (Davidson et al., 2002). This result is not supported by statistical significance, probably because of the small sample size, but it yields interesting clinical information, so its importance should be argued.

In our study, enteropathogenic *E. coli* were more often isolated in 13-21 months-old infants than in 0-12 month-old babies. Our data do not explain the reason for such difference, but we can hypothesize that it could be related to the different type of feeding among the two groups or more probably by attendance at nursery and infant school with the consequent infection risk.

As concerns differences in seasonal prevalence of specific pathogens, epidemic curves need to be considered before proposing probable microorganisms causing acute gastroenteritis in children (O’Ryan et al., 2005). In accord with previous reports conducted in temperate climates, our data describe a strong reduction of acute diarrhea due to enteropathogenic *E. coli* in cold months (Robin-Browne, 1984; Robins-Browne et al., 2004b). The seasonal variation was similar in Rotavirus and Adenovirus and in accord with references (Dennehy, 2000; Staat et al., 2002); Rotavirus still represents the leading winter gastroenteric agent (Dennehy, 2000; Carraturo et al., 2008; Payne et al., 2008).

The present research suggests that diarrheagenic *E. coli* may be an important and unrecognized cause of diarrhea in infancy, not only in developing countries but also in developed areas, such as Italy. Further study of the pathogenic mechanisms of these *E. coli* may have significant implications for the approach to diagnostic and treatment protocols for acute gastroenteritis in children.

A role of primary importance to reduce the diffusion of these pathogenic agents is played by improvements in sanitary measures (i.e. hand-washing after every nappy change and a clean water supply) (Sobel et al., 2004) and food hygiene (Beutin, 2006).

The results of this study allow us to point out the importance of the introduction of potential pathogenic *E. coli* search also in routine diagnostic searches.

The finding of diverse *E. coli* types and particularly the detection of EPEC and ETEC strains stress the need for enhanced surveillance of gastroenteritis agents in infants with more active characterization of the *E. coli* isolated strains.

**REFERENCES**


Caprioli A., Pezzella C., Morelli R., et al. (1996). Enteropathogens associated with childhood diarrhea in Italy. The Italian Study Group on...


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