Original Article

*Escherichia coli* Pathotypes Associated with Diarrhea in Romanian Children Younger than 5 Years of Age

Codruța-Romanița Usein*, Dorina Tatu-Chițoiu’, Simona Cionteaa’, Maria Condei, and Maria Damian

Laboratory of Molecular Microbiology and 1Laboratory of Enteric Bacterial Infections, National Institute of Research-Development for Microbiology and Immunology “Cantacuzino”, Bucharest, Romania

(Received March 27, 2009. Accepted June 5, 2009)

SUMMARY: To document the association of pathogenic *Escherichia coli* with diarrhea in Romanian children, 250 *E. coli* fecal isolates from children under 5 years of age were PCR-screened for well-recognized virulence determinants, as well as for their phylogenetic background. The putative diarrheagenic *E. coli* (DEC) were investigated for susceptibility to various antibiotics. Overall, 61 *E. coli* isolates were classified as enteroaggregative *E. coli* (29 isolates), atypical enteropathogenic *E. coli* (22 isolates), enterotoxigenic *E. coli* (8 isolates), and verotoxin-producing *E. coli* (1 isolate), and one isolate was categorized as unconventional DEC. Only 8 of the PCR-positive isolates would have been assumed to be pathogenic based on their O antigenicity, which highlights the limited effectiveness of serotyping. More than a half (51%) of the pathogenic isolates expressed a multidrug-resistant phenotype, which raises concerns about the therapeutic pediatric approach. The DEC isolates were heterogeneous phylogenetically, deriving from all four major groups: A (31 isolates), B2 (14 isolates), B1 (10 isolates), and D (6 isolates), respectively. Thus, the phylogenetic descent was less significant than the virulence gene content. Our findings document the importance of DEC as a cause of childhood diarrhea in Romania, providing evidence that efforts should be made to estimate the burden of infections by etiology for a better medical approach.

INTRODUCTION

Diarrheal diseases are a leading cause of morbidity and mortality in developing countries and many data regarding the prevalence of various infectious etiologies are based on surveillance studies carried out in these countries. Nevertheless, the burden of intestinal infections concerns the public health systems worldwide, and the need to improve the current evaluation of specific enteric pathogens is irrefutable in any region.

*Escherichia coli* is recognized as an important cause of both sporadic cases and outbreaks of diarrhea all over the world. To date, there are at least six well-characterized classes or pathotypes of *E. coli* that can cause intestinal infections in humans: enteropathogenic *E. coli* (EPEC), Shiga toxin/verotoxin-producing *E. coli* (STEC/VTEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteraggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (1,2). These strains are capable of eluding healthy host defenses due to several specific combinations of virulence factors, but their discrimination from normal fecal flora is not an easy task during routine microbiological diagnostics.

The molecular methods targeting chromosomal or mobile genetic elements coding for virulence traits seem to be the most reliable for diarrheagenic *E. coli* (DEC) identification, but they have not been standardized yet. In our country, the DEC diagnosis is restricted to serological tests for classical EPEC serogroups and the VTEC O157:H7 serotype. Consequently, laboratory reports on the prevalence of the various DEC categories among the causes of intestinal infections are not few in number, but also unreliable in nature. Therefore, the aim of our study was to document the association of pathogenic *E. coli* isolates with diarrhea in Romanian children under 5 years of age, based on the PCR-based identification of their intrinsic virulence. In an era of globalization, each and every effort to improve the surveillance of the various infectious diseases may be regarded as pieces added to complete the puzzle of better controlling them.

MATERIALS AND METHODS

*E. coli* isolates: *E. coli* isolates sent to the Laboratory for Enteric Bacterial Infections in the National Institute of Research-Development for Microbiology and Immunology “Cantacuzino” (NIRDMIC) originated from 250 children under 5 years of age with sporadic diarrhea. The fecal cultures of these children, investigated by the local public health microbiology laboratories during routine work, were negative for *Salmonella*, *Shigella*, and *Campylobacter*. In total, 250 *E. coli* strains (one isolate per child) were investigated.

Serotyping of *E. coli* isolates: All the *E. coli* isolates were serologically typed, using commercially available O antisera for EPEC (O26, O55, O86, O111, O114, O119, O124, O125, O126, O127, O128, O142; BioRad, Marnes-La-Coquette, France) and VTEC O157:H7 (SSI Diagnostica, Hillerød, Denmark) at the Laboratory for Enteric Bacterial Infections from NIRDMIC.

Antimicrobial susceptibility testing: The antimicrobial susceptibility of *E. coli* isolates was determined by the disk-diffusion method according to the Clinical and Laboratory Standards Institute (3). The following antimicrobial agents

---

*Corresponding author: Mailing address: National Institute of Research-Development for Microbiology and Immunology “Cantacuzino”, Molecular Microbiology Laboratory, Splaiul Independenței 103, sector 5, 050096 Bucharest, Romania. Tel: +40215287223, Fax: +40213184414, E-mail: rusein@cantacuzino.ro*
were tested: ampicillin (10 μg), cefotaxime (30 μg), ceftazidime (30 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), trimethoprim-sulfamethoxazole (1.25 + 23.75 μg), streptomycin (10 μg), gentamicin (10 μg), kanamycin (30 μg), tetracycline (30 μg), and chloramphenicol (30 μg). For simplicity, intermediate resistance was classified as resistant. Multidrug resistance (MDR) was defined as resistance to at least three families of tested antibiotics.

Detection of extended-spectrum β-lactamase (ESBL) production: ESBL production was assessed by a double disk synergy method (4) in which the ceftazidime and amoxicillin-clavulanic acid disks were placed 30 mm apart. A clear extension of the edge of the inhibition zone of ceftazidime to indicate ESBL production.

DNA template and PCR for detection of DEC: DNA templates for PCR were obtained from overnight *E. coli* cultures that were pelleted, resuspended in 200 μl of sterile distilled water, and boiled for 15 min. The sequence targets: *eae* ( intimin), *vtx1* (verotoxin 1), *vtx2* (verotoxin 2), *elt* (heat-labile enterotoxin), *est* (heat-stable enterotoxin), and *ipah1* (invasive plasmid antigen) were amplified using DEC Primer Mix (SS Diagnostica), according to the manufacturer’s instructions. For the EAEC detection, the PCR-based protocols targeted the antiaggregation protein transporter gene and the primers developed from the DNA probe CVD432 (5), that targeted the antiaggregation protein transporter gene and the primers developed from the DNA probe CVD432 (5), and the *astA* gene that encodes the EAEC heat-stable toxin 1 (EAST1) (6). In addition, two primer pairs were used to detect the presence of the typical EPEC *bfpA* (bundle-forming pilus) gene (7) and the STEC enterohemolysin-encoding gene *hlyA* (8).

Reference *E. coli* strains (kindly provided by Chantal Le Bouguénec from Pasteur Institute, Paris) were used as PCR positive controls for the genes *bfpA* (EPEC 2349-69), *hlyA* (VTEC EDL933), and *aat* (EAECC 17.2), respectively. *E. coli* strain HB101 served as the negative control for virulence genes in all PCR assays.

Phylogenetic analysis: Isolates were assigned to one of the four main phylogenetic groups of *E. coli* (A, B1, B2, and D), originally defined according to multilocus enzyme electrophoresis (9), using the multiplex PCR-based method of Clermont et al. (10).

**RESULTS**

**Serotyping:** The serological investigation revealed 12 *E. coli* isolates expressing somatic O antigens defining serogroups correlated with EPEC (O26, O55, O86, O112, O126, O128) and VTEC (O157), but the molecular investigation showed that only 8 of them could be considered true DEC based on their virulence gene content. Thus, there were 3 atypical EPEC belonging to serogroups O26, O128, and O157, respectively, 4 EAEC belonging to serogroups O55, O86, and O125, and 1 VTEC belonging to serogroup O126 (Table 1).

**PCR detection of DEC isolates:** From the total of 250 isolates, 61 isolates were PCR-positive for at least one of the targeted virulence genes. The minimal criteria for determination of DEC were as follows: the presence of *elt* and/or *ext* for ETEC, the presence of *vtx1* and/or *vtx2* for VTEC, the presence of *eae* and *bfpA* for typical EPEC, and the presence of only *eae* for atypical EPEC, the presence of *ipah1* for EIEC, and the presence of the *aat* gene as representative for the virulence plasmid pAA of EAEC. Based on these criteria, the 61 isolates were classified as EAEC (29 isolates), atypical EPEC (22 isolates), ETEC (8 isolates), and VTEC (1 isolate).

One strain harboring mixed virulence markers indicative of 2 distinctly defined pathovars (*eae* and *aat*) was categorized as unconventional DEC. The distribution of the other DNA sequences predominantly associated with VTEC (*hlyA*), and EAEC (*astA*) was heterogeneous among the putative diarrhea-causing *E. coli* isolates identified in this study (Table 2). The enterohemolysin-encoding gene was detected in the VTEC isolate, as well as in 2 *E. coli* isolates classified as typical EPEC, while the EAST1-encoding gene was detected in 14 EAEC isolates, 6 ETEC isolates and 9 atypical EPEC isolates, respectively.

**Phylogenetic analysis of *E. coli* fecal isolates:** Overall, 135 (54%) fecal *E. coli* isolates were derived from phylogenetic group A, 56 (22.4%) isolates belonged to group B2, and 37 (14.8%) isolates were assigned to group D. Phylogenetic
group B1 was represented by 22 (8.8%) isolates. Of the 61 putative DEC, 31 isolates belonged to group A, 10 isolates to group B1, 14 isolates to group B2, and 6 isolates to group D, respectively (Table 3).

Antimicrobial susceptibility of DEC isolates: The phenotypic testing of susceptibility of the *E. coli* isolates carrying virulence-associated genes showed that nearly 79% of them (48/61 isolates) were resistant to at least one antibiotic. Actually, 31 of these isolates expressed MDR. Resistance to ampicillin (42 isolates), streptomycin (34 isolates), trimethoprim-sulfamethoxazole (28 isolates), and tetracycline (23 isolates) were the most prevalent resistances, but the patterns among the different *E. coli* pathogenic groups ranged from resistance exclusively to ampicillin or trimethoprim-sulfamethoxazole to resistance to all the antibiotics tested (Table 4). Seven of the multidrug-resistant isolates were confirmed as ESBL producers, and they belonged to ETEC (3 isolates), atypical EPEC (2 isolates), and EAEC (2 isolates) pathotypes, respectively.

### DISCUSSION

In this study, the reference diagnostic approach for DEC isolates was the PCR-based detection of specific genes that render virulence to the microorganisms. In addition, we performed the traditional method of serotyping, targeting exclusively the 12 O serogroups considered by the World Health Organization to represent the EPEC pathotype and VTEC serogroup O157, as usually done during the routine diagnostic of *E. coli* intestinal infections at the laboratory level in our country. As expected, the phenotypic assays widely used in the clinical laboratories with commercial antisera led to false results. The genotyping algorithm increased threefold the diagnostic of true EPEC isolates, and distinguished the pathogenic properties correlated with specific categories of DEC, revealing that 24% of the children with diarrhea were shedding putative DEC.

Previous studies have shown that most of the DEC-positive specimens collected within 48 h after the onset of the gastrointestinal symptoms comprised ≤10% DEC; thus, the colony-based examination may have a low diagnostic sensitivity (11).
Within the present study, a single isolate was investigated for each subject, but regardless of this drawback, the prevalence of *E. coli* isolates carrying virulence-associated genes was surprisingly high. Diarrhea due to pathogenic *E. coli* has been shown to be an important public health issue in developing countries or has been associated with travel to those areas. Our findings showed similarities with the prevalence of DEC infection in children under 5 years of age from Africa (12) and South America (13).

Almost half of the PCR-positive *E. coli* isolates carried virulence genes common to EAEC, which appeared to be the most prevalent DEC category in this study (11.6%). This is in concordance with other reports highlighting the involvement of these microorganisms in sporadic cases as well as outbreaks of diarrhea (14-17). Even though the gold standard for EAEC identification is still considered to be the HEP-2 adherence test, many laboratories resort to PCR-based detection. However, due to the heterogeneity of the adhesins and toxins distinctive of this group of *E. coli*, there is no common genotypic profile for molecular identification. In this study we selected the PCR assay based on the probe CVD432, which has been widely used mainly due to its high sensitivity and specificity (18). Half of the *aat* isolates also possessed the plasmid-borne *astA* gene encoding the toxin EAST1, a 38-amino-acid peptide with homology to the heat-stable enterotoxin of ETEC. As already reported, this category of *E. coli* has emerged as a major etiologic agent associated with diarrhea in children (19-22). In Romania, many healthcare workers are not familiar with EAEC, and there are no reports on the prevalence of this category of *E. coli* in subjects with or without gastrointestinal symptoms. A collection of intestinal isolates of *E. coli* originating from the fecal culture of 34 healthy children under 3 years of age (3 colonies per child) used as a control lot in a study of uropathogenic *E. coli* isolates from children was screened for the presence of pAA. Only one child shed *aat* isolates (Codoruța-Romania Usein, unpublished observation), suggesting that EAEC may not be endemic in Romania. However, a regular monitoring of healthy children and adults would be helpful in a survey of *E. coli* strains carrying markers of EAEC. This would also be very important for the epidemiology of EPEC infection. In this study, almost 9% of the children with diarrhea carried EPEC isolates; all were atypical EPEC because they possessed only the virulence marker *eae*, indicative for the presence of the “locus of enterocyte effacement” (LEE) pathogenicity island, but lacked the marker for the EPEC adherence factor (EAF) plasmid. In addition, some of these isolates possessed non-LEE-encoded virulence factors. Most of them were positive for *astA*. In a previous study, reporting a similar prevalence of atypical EPEC as found in our collection, the EAST1 was considered a potential virulence marker for the diagnosis of truly atypical EPEC pathogenic strains (23), while others proposed a PCR-based protocol including *astA* as a target in order to improve the diagnosis of both typical and atypical EAEC, particularly in outbreak settings (24).

Atypical EPEC is seen as a heterogeneous group comprising members differing in their virulence potential. Particular lineages may be closely related to VTEC, sharing certain virulence determinants. In this study, 2 *eae*+ *E. coli* isolates harbored the genes encoding enterohemolysin, which are located in the 60-Mda plasmid found in nearly all O157:H7 strains and also widely in non-O157 VTEC strains. At the same time, the *E. coli* O157:H7 isolate exclusively harbored the *eae* gene. These results might be due to the loss of the verotoxin-encoding genes during the course of infection, or they might be indicative of intrinsic verotoxin-negative variants. It seems that the first may still cause severe disease, while the latter cause mostly uncomplicated diarrhea (25).

In our study only one child was diagnosed with VTEC infection. The sorbitol-fermenting VTEC isolate, which was assigned to serogroup O126, harbors *vtx2* alone, as well as LEE-associated *eae*, and the gene *hlyA*. It is worth noting that this serogroup was not frequently reported as comprising VTEC isolates, and was associated with non-bloody diarrhea (26). The first Romanian report regarding the presence of VTEC non-O157 isolates from humans with diarrhea indicated isolates belonging to other classical EPEC serogroups, namely O26, O55, and O128 (27).

The ETEC isolates were less prevalent (3.2%) among the Romanian children screened for shedding DEC, which is in concordance with previous reports signaling the high prevalence of this pathotype in developing countries (1,28). In addition, the absence of EIEC isolates could suggest the limited role of both DEC categories in childhood diarrhea in Romania.

When evaluating the pathogenic potential of each phylogenetic group, a significant association with diarrhea was observed for phylogenetic group B1; almost half (45%) of the children’s isolates assigned to this group carried virulence determinants. Groups B2 and A concentrated lesser DEC isolates (25 and 23%, respectively), while only 16% of the isolates assigned to group D harbored virulence traits. Previous studies showed that a specific genetic background is required for acquisition and expression of virulence factors in *E. coli* (29,30). In our study, the detection of specific virulence genes was more useful than phylotyping due to the fact that the majority of pathogenic isolates were EAEC and atypical EPEC, and as already reported by other researchers, these pathotypes are phylogenetically heterogeneous (30). Nevertheless, we also obtained some results that disagree with the concept that VTEC and ETEC are found outside groups B2 and D (29). Larger studies on pathogenic *E. coli* isolates representing the diversity of the species might help clarify these issues.

Owing to changing patterns of antimicrobial resistance, knowledge of recent regional patterns is critical to therapeutic decision-making. An important finding of this investigation was that more than half (64.5%) of the DEC isolates shed by children were multi-resistant, which raises concerns about the pediatric therapeutic options. Almost 46% of the isolates co-expressed resistance to ampicillin, trimethoprim-sulfamethoxazole, and streptomycin. In Romania, antibiotic use is not properly monitored, and there is a strong tendency for local pediatricians to prescribe antibiotics to children without taking into account the development of resistant strains. A possible explanation for the high rates of DEC resistance to ampicillin (68.8%), trimethoprim-sulfamethoxazole (45.9%), and tetracycline (40.0%) might be the large local intake of these antibiotics, resulting in high selective pressure. However, this speculation does not apply to streptomycin, which has had limited clinical use for many years; nonetheless, the high rate of resistance (70.8%) to this drug has persisted. This issue was previously reported not only in relation to streptomycin, but also to sulphonamides (31). Possible explanations for the persistence of resistance to these drugs include the properties of the mobile elements on which the determinants are carried, and the potential selection pressures other than antibiotics for human medical use. The MDR expressed
among the studied DEC collection included resistance to antibiotics which are not recommended for children, such as fluoroquinolones, chloramphenicol, tetracycline, and kanamycin. This resistance could be due to environmental conditions, such as transmission of resistant isolates between adults and children, or between animals and humans. The latter implies the use of antimicrobials in food animals, resulting in the selection of resistant bacteria that are transported by the food vehicle.

Infections due to ESBL-producing *E. coli* seem to be increasing all over the world (32-34). In our study, 11% of the DEC isolates were resistant to third-generation cephalosporins (www.rivm.nl). In the same year, 16% of the *E. coli* isolates from various intestinal and extraintestinal specimens were identified as ESBL producers in the Laboratory for Enteric Bacterial Infections in NIRDMIC (unpublished data). In order to evaluate the real significance of this issue for the public health, well conducted national surveillance studies are needed.

In conclusion, our findings point to the potentially underestimated role of *E. coli* in causing diarrhea. It is obvious that efforts should be made, including the improvement of clinical laboratory methodology, to estimate the burden of infections by etiology at regional levels, in order to better orient a national strategy for prevention and control of human infections in Romania.

REFERENCES