Original Article

Genotypic and phenotypic features of enteropathogenic *Escherichia coli* isolated in industrialized and developing countries

Simona Santona[1], Nicia Diaz[1], Pier Luigi Fiori[1,2], Moises Francisco[3], Mohsin Sidat[4], Piero Cappuccinelli[1,2], Paola Rappelli[1,2]

[1]*Department of Biomedical Sciences, University of Sassari, Sassari, Italy*
[2]*Center of Excellence for Biotechnology Development and Biodiversity Research, University of Sassari, Italy*
[3]*Department of Microbiology, University of Luanda, Luanda, Angola*
[4]*Department of Community Health, "E. Mondlane" University, Maputo, Mozambique*

Abstract

Introduction: Typical EPEC are considered a leading cause of diarrhoea in developing countries, while atypical EPEC have been isolated more frequently in developed areas. The actual geographic distribution of the two EPEC subgroups is controversial, since data can be highly influenced by laboratory resources. This study aimed to compare the distribution of typical and atypical EPEC among children in developed and developing countries, and to characterize the bacterial isolates, using a unique methodological approach.

Methodology: A total of 1,049 *E. coli* were isolated from faeces of children with acute diarrhoea in Mozambique, Angola and Italy, and processed by PCR to assess the presence of a large panel of virulence genes. All isolates classified as EPEC were further characterized by evaluating adherence and capability to induce actin rearrangement on Hep-2 cells.

Results: Overall we isolated 59 EPEC, likewise distributed in the three countries, representing the 5.04%, 4.44% and 6.97% of all Mozambican, Angolan and Italian isolates, respectively. Nevertheless, the geographic distribution of the two EPEC subgroups was not homogeneous: in Italy we isolated 28 aEPEC but no tEPEC, while in Angola and Mozambique the percentage of the two subgroups was comparable. Twelve atypical EPEC were FAS positive and able to induce localized-like adherence on Hep-2 cells, but no correlation with the geographic origin of isolates was observed.

Conclusion: Atypical EPEC are present in sub-Saharan areas in a percentage similar to that of typical strains, and are not mainly restricted to industrialized countries, as it was previously supposed.

Key words: diarrhoeagenic *E. coli*; atypical EPEC; Italy; Mozambique; Angola

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Introduction

Escherichia coli is a common inhabitant of the human gut, where it acts as a harmless component of the normal flora. In some cases, however, it can be responsible for severe diarrhoea, especially in children. On the basis of their virulence traits, diarrhoeagenic *E. coli* are classified in pathotypes, named enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EAggEC), diffusely adherent (DAEC), enterohemorrhagic (EHEC), and enteropathogenic *E. coli* (EPEC) [1]. Enteropathogenic *E. coli*, which are the first to be recognized as a leading cause of infantile diarrhoea, do not produce toxins and are not invasive [2]. Their primary pathogenetic mechanism is a lesion called "Attaching and Effacing" (A/E), characterized by microvilli destruction [3]. The lesion is the consequence of an intimate adherence of *E. coli* to the intestinal epithelium, followed by the aggregation of target cell actin and other cytoskeletal proteins at the site of bacterial attachment, resulting in the formation of the typical pedestal [4]. Genes necessary for the establishment of A/E lesions are located on a chromosomal 35-kb pathogenicity island called the "locus of enterocyte effacement" (LEE) [5]. It harbours several genes involved in pathogenicity, including *eae,* which codes for intimin, a bacterial outer membrane protein that mediates the intimate adhesion between bacteria and enterocytes [6].

Enteropathogenic *E. coli* are able, *in vitro,* to adhere to epithelial cells in a pattern named "localized adherence" (LA), that is dependent on the presence of the EAF (EPEC adherence factor) plasmid, harboring the gene coding for a type IV pilus called "bundle

forming pilus" [7]. However, some strains lack the EPEC adherence factor plasmid, and are able to adhere to target epithelial cells only after an extended period of coincubation, usually showing a characteristic pattern called "localized adherence-like" (LAL). On the basis of the presence of the EAF, plasmid EPEC strains recently have been divided in two subgroups: typical EPEC (tEPEC) that harbor the plasmid, and atypical EPEC (aEPEC) that do not possess it [8].

In developed countries, infections caused by typical EPEC are considered of limited impact, and usually present in the form of sporadic outbreaks [9]. On the contrary, in developing countries, enteropathogenic *E. coli* are a major cause of infant diarrhoea, especially in the age group ranging from 0 to 6 months [10]. In recent years, the incidence of diarrhoea due to typical EPEC strains in developing countries appears to have decreased slightly. It is not clear whether it is only a result of the improved conditions of infants' health, or it is rather the consequence of a more accurate etiologic identification of diarrhoeal episodes [11]. In fact, while at first the identification of EPEC was mainly based on serotyping, it is now clear that this approach can lead to overestimate enteropathogenic *E. coli*, and that they are better identified on the basis of their genotypic features.

Whether atypical EPEC is associated with diarrhoea remains controversial, but recent data seem to indicate aEPEC as an emerging cause of gastrointestinal disease. Unlike typical EPEC, atypical EPEC seem to be more common in industrialized countries, even if recent studies emphasize their diffusion also in developing regions [12]. Nevertheless, limited data are available on the real geographical distribution of aEPEC, probably because they are not always distinguished from the typical forms in epidemiologic studies, especially in countries with limited resources. In fact, the two subgroups of enteropathogenic *E. coli* are distinguishable only by expensive molecular methods, able to differentiate them on the basis of the presence/absence of the *bfpA* gene.

The present work aimed to investigate the diffusion of typical and atypical EPEC in both developed and developing countries, and to define the phenotypical and genotypical features of bacterial isolates, using an single methodological approach, thus avoiding any bias due to various identification techniques often applied in different geographic regions. The study has been performed on three homogeneous groups of children of less than five years of age from Italy, Angola, and Mozambique, suffering from acute diarrhoea.

Methodology

Patients and strains

The *E. coli* strains examined in this report were isolated in Mozambique, Angola, and Italy from children with diarrhea (defined as at least three loose movements in the previous 24 hours) younger than 5 years of age. Three hundred seventy-seven strains were isolated in Mozambique from March 1998 to March 1999 at the laboratory of Microbiology at the Faculty of Medicine, University of Maputo. *E. coli* from Angola (total 270) were isolated in the refugee camp of Funda, from April 1996 to March 1997, and 402 Italian strains were from children attending the Paediatric Division of the University of Sassari between 1997 and 1999.

Several *E. coli* strains were used as controls: E-A37 (ETEC st^+/ lt^+), E-A29 (ETEC st^+/ lt^-), E-F1 (EPEC eae^+/ $bfpA^+$), and E-D21 (EHEC eae^+/ stx_1^+/ stx_2^-) were kindly provided by Alfredo Caprioli, Istituto Superiore di Sanità, Italy; EIEC strain X6171 was a gift from Roy Curtiss III, St. Louis, USA; atypical EPEC *E. coli* SS173 (eae^+/ $bfpA^-$) and the non-pathogenic HB101 were from our own collection.

PCR

E. coli were isolated on MacConkey agar plates, then three colonies, tentatively identified as *E. coli*, were inoculated in Luria Bertani (LB) broth and incubated overnight at 37°C. To extract DNA, *E. coli* from 50 µl of overnight culture were added to 450 µl of sterile distilled water, and boiled for 20 minutes. Each sample was then centrifuged at 14000 x g for 10 minutes and the supernatant stored at -20°C until used. *E. coli* isolated in Angola and in Mozambique were inoculated in LB plus 0.8% agar, then transported to the microbiology laboratory of the University of Sassari and processed as described above.

All *E. coli* isolates were characterized by a set of three multiplex PCR to assess the presence of virulence genes as described in detail elsewhere [13]. Briefly, 1 µl of bacterial DNA, obtained as described above, was mixed with 0.35U of Taq polymerase (Invitrogen, Milan, Italy), 0.5 µM of each primer, 200 µM of deoxynucleoside triphosphate (Invitrogen, Milan, Italy) and PCR Buffer 10x (50mM KCl, 10mM Tris-HCl, 1,5mM MgCl2) in a final volume of 15 µl, then subjected to 35 cycles consisting of one minute of denaturation (94°C), one minute of annealing (58°C), and one minute at 72°C. Amplification products were

electrophoresed through a 1% agarose gel and visualized with a UV transilluminator after ethidium bromide staining. Strains that tested positive for *eaeA* gene but negative for genes stx_1 and stx_2 were selected for further characterization. Results were analyzed using Fisher's exact test.

Adhesion to epithelial cells

An adhesion assay was performed on epithelial cells, according to the technique described by Cravioto *et al*. [14]. Hep-2 monolayers were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) in 24-well plates containing round glass coverslips to a 70-80% confluence. Cells were then incubated in RPMI 1640 (2% FBS) added with 0.5% D-mannose for 15 minutes at 37°C and 5% CO_2. Bacteria were grown overnight in Luria-Bertani broth, then washed in PBS buffer, resuspended at a concentration of 10^8/ml in RPMI 1640 plus 2% FBS, and added to washed epithelial cells. After three and six hours of co-incubation at 37°C cells in 5% CO_2, cells were extensively washed with PBS to remove non-adherent bacteria, fixed with methanol, then stained with May-Grumwald-Giemsa (1:20 vol/vol) for 20 minutes. Coverslips were finally washed with H_2O and examined by light microscopy. The adherence patterns were classified as localized (LA), localized adherence-like (LAL), aggregative (AA), diffuse (DA), or negative adherence (NA).

FAS test

The ability of bacteria to aggregate actin *in vitro* was investigated by means of the Fluorescence Actin Staining test (FAS test) as described previously by Knutton *et al*. [15]. FAS test was performed on Hep-2 cells seeded on glass-coverslips in 24-well plates. Overnight grown bacteria were added to epithelial cells in RPMI 1640 medium supplemented with 2% FBS and 0.5% D-mannose, then incubated at 37°C in 5% CO_2. After three and six hours of infection, monolayers were washed with PBS, fixed in 3% paraformaldehyde and permeabilized in 0.25% Triton X-100. Then, after washing three times in PBS, cells were stained with 5 µg/ml of fluorescein isothiocyanate (FITC)-labeled phalloidin (Sigma-Aldrich, St. Louis, USA**)** and 0.2 µg/ml of 4',6-diamidino-2-phenylindole (DAPI) for 15 minutes [16]. Coverslips were then washed, mounted onto slides, and examined by fluorescence microscopy.

Results

In this report we describe the prevalence and the virulence profile of typical and atypical EPEC strains isolated from stools of children with acute enteritis in different geographic areas.

We examined a total of 1,049 *E. coli* strains, which were isolated from children with diarrhoea in both developed (Italy) and developing countries (Mozambique and Angola). All isolates were characterized by multiplex PCR to define the pathotype, and results are shown in Table 1. *E. coli* identification was confirmed using primers for the species-specific gene *uidA*. As shown, even if the overall percentage of diarrhoeagenic strains in the different countries is almost equivalent, the distribution of single pathotypes differs greatly. ETEC strains, for instance, represent the 4.51% of all *E. coli* isolated in Mozambique and the 6.3% of Angolan isolates, but in Italy they have been isolated in only one patient. On the contrary, EHEC strains were found in Italy (2.74%) but not in sub-Saharan regions.

All strains presenting the *eaeA* gene for intimin but

Table 1. Distribution of diarrhoeagenic *E. coli* pathotypes among bacterial strains examined

	Mozambique (n = 377)		**Angola (n = 270)**		**Italy (n = 402)**	
	positives	%	positives	%	positives	%
ETEC	17	4.51	17	6.30	1	0.25
EPEC	19	5.04	12	4.44	28	6.97
EHEC	0		0		11	2.74
EIEC	12	3.18	1	0.37	0	
TOTAL	**48**	**12.73**	**30**	**11.11**	**40**	**9.95**

not the stx_1 or stx_2 for shiga-like toxins, therefore classified as EPEC, were further characterized. A total of 59 *eaeA* positive/*stx* negative strains were tested for the presence of the *bfpA* gene, coding for the binding-forming pilus, and thus divided accordingly in typical and atypical EPEC. Interestingly, in Italy, no typical EPEC were found and all 28 enteropathogenic strains were classified as atypical EPEC, representing the 7.0% of the total *E. coli* isolated, while both in Angola and Mozambique the percentage of aEPEC dropped to 2.6 % of isolates. Table 2 shows the percentage of aEPEC and tEPEC isolated in the different geographic areas: the overall distribution of aEPEC and tEPEC in developing and in developed countries is significantly different (*p value* < 0,0001).

Hep-2 adhesion assay

All typical and atypical EPEC strains were subjected to an adherence assay on HEp-2 cells. Each bacterial strain was incubated with epithelial cells for three and six hours. Adherence patterns were classified as follows: localized adherence (LA) when bacteria form tight clusters on the target cell surface within three hours, and localized-like adherence (LAL) when loose clusters are formed only after six hours of co-incubation; diffuse adherence (DA); and aggregative adherence (AA), when bacteria stick to cells with a stacked brick pattern.

We analyzed a total of 14 typical EPEC, and all of them showed the expected localized adherence within three hours of contact with epithelial cells. Conversely, none of the 45 aEPEC analyzed showed a localized adhesion within three hours of incubation. When incubation was extended up to six hours, a localized-like adherence phenotype was observed in 17 isolates (37.7% of total aEPEC). Eighteen isolates showed a DA phenotype (40%), three isolates showed an irregular or indeterminate adherence phenotype (6.6%), and seven isolates were not able to adhere to target cells (15.5%).

FAS test

The 14 tEPEC and the 45 aEPEC strains were investigated for their ability to induce A/E lesions on target cells by the FAS test. The characteristic accumulation of actin at the site of bacterial adhesion was observed in all 14 typical EPEC after three hours of contact. None of the 45 atypical EPEC was able to induce visible actin rearrangements within three hours, but 12 of them turned positive to the FAS test after six hours of coincubation, showing the typical pedestal on HEp-2 cells. DAPI was used to highlight bacteria on the surface of target cells during fluorescence experiments, and confirmed the patterns observed in the adhesion assay. A positive DAPI staining was observed for all adhering *E. coli* and demonstrated that when negative FAS test results were obtained they were really due to the incapacity of some *E. coli* strains to induce actin rearrangements, and not to a lack of adhesion.

Discussion

In the present work we focused on the occurrence and properties of typical and atypical EPEC in children with diarrhoea in both developed and developing countries, using a unique methodological approach. We analyzed 1049 *E. coli* strains isolated from the faeces of a homogeneous population of symptomatic children of less than five years of age living in Italy, Angola, and Mozambique.

Isolates have been characterized and divided in pathotypes on the basis of their virulence genes. Results show that the percentage of diarrhoeagenic *E. coli* in the three countries was comparable, ranging from 9.95% in Italy to 12.73% in Mozambique, but as expected, the distribution of the different pathotypes was not homogeneous. In fact, our data confirmed what we have already highlighted in other studies [17,18], and show that in Angola and in Mozambique no enterohaemorrhagic *E. coli* are present, in contrast to what happens in Italy, where a large number of

Table 2. Distribution of typical and atypical enteropathogenic *E. coli* in the different geographic areas

	Mozambique		**Angola**		**Italy**	
	positives	%	positives	%	positives	%
Typical EPEC	9	47.3	5	41.7	0	0
Atypical EPEC	10	52.6	7	58.3	28	100

EHEC, corresponding to the 2.74 % of *E. coli* isolates, has been identified. As expected, ETEC strains are present in Mozambique and Angola in a high percentage (4.51% and 6.3%, respectively), while in Italy they have been isolated in only one patient.

A total of 59 EPEC strains were classified as typical and atypical of the basis of the presence of the *bfpA* gene. Overall, the percentage of EPEC did not vary greatly among the three countries, ranging from 4.44% to 6.97%, values that are consistent with those obtained in previous studies on symptomatic populations [12,19,20]. Nevertheless, when analyzing the isolates on the basis of the presence of the *bfpA* gene, we observed an interesting difference in the distribution of atypical and typical EPEC strains. A total of 45 aEPEC and 14 tEPEC were isolated in the three countries. In Italy, we found 28 aEPEC but no typical EPEC, confirming that their circulation in this country is very scarce [21,22]. Data obtained from Italian strains are in accordance with those obtained in other European countries such as England, where among 2,774 *E. coli* samples isolated from symptomatic children, 142 strains were identified as atypical EPEC and only one as typical EPEC [23], and Romania, where about 9% of isolated *E. coli* were classified as atypical EPEC but no typical EPEC were found [24]. On the contrary, in the sub-Saharan countries we found that typical and atypical EPEC were present in a comparable ratio. This finding is in agreement with data recently obtained in epidemiological studies in developing countries [12,25,26]. In fact, it is becoming clear that atypical EPEC are diffused also in these areas, and our results confirm this finding.

To investigate the potential pathogenetic properties of isolated strains, we studied their adhesion features and their capability to induce A/E lesion *in vitro* on HEp-2 cells. Typical EPEC strains normally show a characteristic LA pattern in adhering assays, while atypical EPEC can show LAL, DA, AA patterns, usually only after a prolonged co-incubation time [27,28]. In this work, as expected, only the typical EPEC strains, all isolated in the sub-Saharan area, gave the classical localized adherence within three hours of contact with the epithelial target cells, and atypical EPEC strains showed different patterns of adhesion, when co-incubation with HEp-2 cells was extended up to six hours. The LAL pattern was observed in 17 aEPEC isolates, and 18 showed a diffuse adherence. Three atypical strains adhered in an irregular and indeterminate way, and seven did not adhere at all. We did not observe any correlation between the adhesion pattern and the geographic origin of bacterial strains. All isolated *E.coli* were then subjected to the FAS test, to highlight the capability to induce A/E lesions on target cells. We observed that only the 14 typical EPEC, all showing the LA adhesion pattern, gave positive results for the FAS test after three hours. Among the atypical strains, for which the coincubation with HEp-2 cells was prolonged to six hours, only 12 were able to induce actin rearrangements at the site of contact with the target cells. All of them showed a localized-like adhesion adherence pattern, suggesting that they may possess virulence determinants that can replace the bundle forming pilus in the first interaction with target cells. No correlation with the geographic region of origin was observed: eight were isolated in Italy, two in Mozambique and two in Angola, representing about one third of the total aEPEC isolated in each country. Recent studies indicate that LAL and FAS test positivity represent important virulence properties of atypical EPEC strains, and indicate their potential role as causative agents of diarrhoea. In fact, even if they do not possess the bundle forming pilus, it appears that they can express other adhesive structures, able to replace it in the first phases of adhesion [28].

Conclusion

The present study demonstrates that atypical EPEC are present in sub-Saharan areas in a percentage similar to that of typical strains, confirming the more recent observations of several research groups that subvert the initial theory that aEPEC were diffused mainly in industrialized countries. The analysis of virulence features of isolates confirms that aEPEC can play a role in the etiology of infant diarrhoea, and that further studies are needed to shed light on their pathogenic mechanisms.

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Corresponding author
Paola Rappelli
Department of Biomedical Sciences
Viale S. Pietro 43/B, 07100 Sassari, Italy
Telephone: ++39079228299
Fax: ++39079212345
Email: rappelli@uniss.it

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