

## Pathogenic enteric *Escherichia coli* in children with and without diarrhea in Maputo, Mozambique

Paola Rappelli <sup>a</sup>, Elena Folgosa <sup>b</sup>, Maria Letizia Solinas <sup>a</sup>, Joao Leopoldo DaCosta <sup>c</sup>,  
Claudia Pisanu <sup>a</sup>, Moshin Sidat <sup>c</sup>, Josefa Melo <sup>b</sup>, Piero Cappuccinelli <sup>a,d</sup>,  
Mauro Maria Colombo <sup>d,e,\*</sup>

<sup>a</sup> Dipartimento di Scienze Biomediche, Divisione di Microbiologia Sperimentale e Clinica, Università di Sassari, Viale S. Pietro 43/B, Sassari, Italy

<sup>b</sup> Departamento de Microbiologia, Faculdade de Medicina, Universidade E. Mondlane, Av. S. Allende, Maputo, Mozambique

<sup>c</sup> Departamento de Saude da Comunidade, Faculdade de Medicina, Universidade E. Mondlane, Av. S. Allende, Maputo, Mozambique

<sup>d</sup> Consorzio Interuniversitario per la Cooperazione con i Paesi Emergenti (CICUPE), Università di Roma la Sapienza, Piazzale A. Moro 5, Roma, Italy

<sup>e</sup> Dipartimento di Biologia Cellulare e dello Sviluppo, Università di Roma la Sapienza, Via dei Sardi 70, Roma, Italy

Received 10 March 2004; received in revised form 8 July 2004; accepted 9 July 2004

First published online 29 July 2004

### Abstract

A study was conducted on the circulation of potentially diarrheagenic *Escherichia coli* in two groups of children, both under the age of seven. The first group (548 children) suffered from mild diarrhea and attended the Xipamanine Health Center of Maputo, in Mozambique. The second group (380 children) included randomly chosen, asymptomatic, children from the same population. A total of 503 *E. coli* strains were isolated from the two groups of children ( $n = 375$  and 128, respectively). All *E. coli* strains were genotypically and phenotypically screened. The presence of virulence-associated genes was assessed by a set of multiplex PCR specific for *st* and *lt* genes of enterotoxigenic *Escherichia coli* (ETEC), *eae* and *hfpA* genes of enteropathogenic *E. coli* (EPEC), *stx*<sub>1</sub> and *stx*<sub>2</sub> of enterohemorrhagic *E. coli* (EHEC), *ial* of enteroinvasive *E. coli* (EIEC) and the species-specific gene *uidA*. Adhesion and cytotoxicity of isolated *E. coli* were evaluated in vitro on different cell cultures. A total of 37 isolates harbored virulence-associated genes: 18 were classified as ETEC, (15 from symptomatic, and three from asymptomatic children), 16 as EPEC (respectively, 13 and 3) and three EIEC in the symptomatic group. No *stx*<sub>1</sub> or *stx*<sub>2</sub> genes, associated with enterohemorrhagic *E. coli* were found. On the basis of the adhesion pattern on HeLa cells, 167 *E. coli* were classified as diffusely adhering, (125 in patients and 42 in controls) and 67 as enteroaggregative, (50 and 17, respectively). To the best of our knowledge, this is the first report in the literature on the circulation of potentially diarrheagenic *E. coli* in Mozambique.

© 2004 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

**Keywords:** Diarrheagenic *E. coli*; Cell adherence; PCR identification; Mozambique

### 1. Introduction

Diarrhea is one of main sources of morbidity worldwide and a large proportion of cases is caused by diar-

rheagenic *Escherichia coli* [1]. The circulation of different pathogenic *E. coli* is an important problem in developing countries, enhanced by many factors, such as climatic adversities, poor sanitation, malnutrition and AIDS related immunodepression. These high risk social-economic parameters lead to a favorable environmental condition for the emergence of new enteropathogenic organisms or strains especially in childhood.

\* Corresponding author. Tel.: +39 06 49917585; fax: +39 06 49917594.

E-mail address: mauro.colombo@uniroma1.it (M.M. Colombo).

Monitoring and characterizing *E. coli* strains, relating to clinical symptoms, is crucial for epidemiological control, in order to survey the insurgence of genetic rearrangement of virulence factors and track the emergence of new pathogenic strains [2].

The epidemiological impact of each *E. coli* pathotype in childhood diarrhea varies with the geographical area around the world and specifically in Africa [3–7]. No studies have yet investigated the etiology of mild diarrhea in Mozambique, with particular attention to *E. coli*, and with both molecular and cytological approaches.

The different classes of diarrheagenic *E. coli* can be identified by the presence of genes coding for specific virulence factors that are absent in non-pathogenic strains. There are now at least seven pathotypes [1] classified on the basis of their clinical features and virulence properties. These are the enteropathogenic (EPEC), enterotoxigenic (ETEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), and the most recently identified enteroaggregative *E. coli* (EAEC), diffusely adhering *E. coli* (DAEC) and cytolethal distending toxin (CDT)-producing *E. coli*. Of these, EPEC, ETEC, EIEC, EHEC and EAEC are clearly associated with different clinical types of enteritis, while the other pathotypes are candidates as potential pathogens but their association with diarrhea has not been clearly assessed, and further studies are required to confirm their etiological role in enteric diseases [8].

The identification of diarrheagenic *E. coli* cannot be based only on cultural and biochemical criteria, since they are indistinguishable from the non-pathogenic *E. coli* commonly found in human feces. Moreover, specific serotyping is not always correlated with pathogenicity. The discrimination of ETEC, EPEC, EHEC and EIEC strains requires DNA target based screening by molecular techniques. Several PCR protocols, with both single and multiple target genes, have been recently proposed to identify each specific pathotype of diarrheagenic *E. coli* [9,10]. Each protocol has different features and needs specific procedures. In our study, we apply a set of multiplex PCR for the simultaneous and specific identification of enterotoxic, enterohemorrhagic, enteropathogenic and enteroinvasive *E. coli* [11]. Since enteroaggregative, diffusely adhering and toxin-producing *E. coli* are not defined by the presence of unique virulence genes, we based their definition on their adhesion to HeLa cells, and on their cytotoxic activity for cell cultures *in vitro*. In fact they represent the most disputed diarrheagenic pathotypes, in which clear-cut virulence genes have not been identified so far; these still need to be better correlated with clinical evidence to ascertain their real pathogenic potential [8–12]. Since it is crucial to investigate and exclude the co-occurrence of other enteric pathogens, such as parasites and rota-

virus, particular attention has been paid to their diagnosis. The present study, that lasted from March 1998 to March 1999, was undertaken to follow the circulation of potentially pathogenic *E. coli* among children under seven years with mild diarrhea in Mozambique. All children, were living in Xipamanine, a suburban neighborhood of Maputo, characterized by poor health and social conditions. This study focuses on the isolation of pathogenic *E. coli*, causing mild or atypical diarrhea, in order to better understand the disease inducing role of disputed pathotypes. During the period of the study, all the cases of severe diarrhea were referred to the cholera diagnostic unit, because of an ongoing cholera epidemic, and were therefore excluded.

## 2. Materials and methods

### 2.1. Sample collection

Stool samples from 548 children with mild diarrhea (Group 1) attending the Xipamanine Health Center from March 1998 to March 1999 were collected and transported to the laboratory of Microbiology at the Faculty of Medicine of the University of Maputo. Individual forms were filled with identification data and description of fecal consistency and the presence of mucus or blood. Samples were then processed for microscopic examination for leucocytes and erythrocytes, parasitological examination and Rotavirus testing. A second group of 380 asymptomatic children (controls) were randomly selected in the same urban area and stool samples were collected and submitted to the same procedures. Children were not subjected to any antibiotic therapy in the week preceding the sampling. Informed consent was obtained from each child's parent.

### 2.2. Pathogen identification

Stool samples were microscopically examined for trophozoites and cysts of protozoa and intestinal helminth eggs and larvae, according to the following procedures: wet mounts both in saline and in iodine solution, modified Ziehl-Neelsen and trichromic staining of smears, and Ritchie concentration technique. Rotavirus antigens were detected in the stools by the Elisa Premier Rotaclone kit (Cambridge Biotech, Worcester, MA), according to the manufacturer's instructions. *E. coli* strains were isolated by culturing stool samples on solid media (XLD and MacConkey); preliminary identification was confirmed by API 20 Esystem (BioMerieux, Marcy L'Etoile, France). Isolated strains were grown in Luria Bertani (LB) broth, then frozen at  $-80^{\circ}\text{C}$  for further procedures.

### 2.3. PCR assays

To extract DNA, *E. coli* from 50 µl of overnight culture were pelleted, resuspended in 500 µl of sterile distilled water, and boiled for 20 min. Samples were then centrifuged at 14,000g for 10 min and the supernatants were stored at –20 °C until further use. Each DNA sample was subjected to a set of three multiplex PCR assays described in detail elsewhere [12]: the first assay utilizes primer pairs specific for *st* and *lt* genes of ETEC, and for the gene coding for *E. coli* β-glucuronidase (*uidA*), used as a control; assay 2 detects the presence of the *eaeA* and *bfpA* genes of EPEC, and assay 3 recognizes *stx*<sub>1</sub> and *stx*<sub>2</sub> of EHEC, and *ial* of EIEC.

*Escherichia coli* control strains E-A37 (ETEC *st*<sup>+</sup>/*lt*<sup>+</sup>) and E-F1 (EPEC *eae*<sup>+</sup>/*bfpA*<sup>+</sup>) were kindly provided by A. Caprioli (Istituto Superiore di Sanità, Italy), X6171 (EIEC *ial*<sup>+</sup>) was provided by R. Curtiss III (St. Louis, MO), and SS115 (EHEC *eaeA*<sup>+</sup>/*stx*<sub>1</sub><sup>+</sup>/*stx*<sub>2</sub><sup>+</sup>) and the non-pathogenic *E. coli* HB101 were from our own collection.

All oligonucleotide primers were obtained from a commercial source (Life Technologies, Inc.). For each multiplex reaction 1 µl of DNA preparation was amplified in a 25 µl volume containing 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.1 Triton X-100, 200 µmol of each desoxyribonucleotide, 10 pmol of each primer and 0.5 units of DynaZyme thermostable DNA polymerase (FynnZymes, Oy, Finland). A single cycling protocol was used for all multiplex PCR assays, with the following parameters: denaturation for 5 min at 94 °C, then 30 cycles of amplification (denaturation 1 min at 94 °C, annealing 1 min at 58 °C and extension 1 min at 72 °C). Amplified samples were electrophoresed on 1% agarose gel containing ethidium bromide (1µg/ml), and visualized by UV transillumination.

### 2.4. Adhesion to epithelial cells

Epithelial HeLa cells were cultured in RPMI 1640, 10% FBS medium, in 24-well plates containing a round coverslip inside each well. Overnight grown bacteria were washed in PBS buffer, resuspended in RPMI 1640 medium and added to washed cell cultures at a 50:1 ratio. After 3 h of incubation the coverslips were washed, stained with Giemsa for microscopic observation of adhesive patterns, and classified as displaying localized (LA), aggregative (Agg) or diffuse (DA) adhesion [13].

### 2.5. In vitro cell cytotoxicity assay

VERO and CHO cells were cultured as described above. Bacteria were grown in LB broth overnight and

centrifuged at 3500 rpm for 15 min. Supernatant was filtered through a 0.45 µm millipore filter and added to the washed cell cultures in a 96-well plates at 1:20 ratio with culture medium. The effect of bacterial supernatant was observed after 24, 48 and 72 h. All experiments were performed in triplicate.

## 3. Results

Rotavirus antigens were detected in 18.2% of symptomatic (100/548) and in 5% of asymptomatic children (19/380), *p* < 0.0001. Parasites were identified in 37.8% of cases (207/548) and in 56.8% of healthy controls (216/380), and their presence as a whole was not statistically associated with diarrhea. In the group of 548 children with diarrhea, a total of 375 *E. coli* (68.4%) were found, and from the 380 healthy controls 128 (33.7%) isolates were obtained (*p* < 0.0001), indicating an alteration of intestinal flora in the first group. All *E. coli* isolates were subjected to three multiplex PCR tests to assess their genotypic profile of pathogenicity and all 503 generated positive *uidA* results, confirming species identification. Of the 503 *E. coli*, 298 showed a potentially pathogenic phenotype or genotype, of which 229 (41.8%) came from symptomatic and 69 (18.1%) came from asymptomatic children, as shown in Table 1(A). Statistical analysis indicates that the difference between the two groups was highly significant (*p* < 0.0001).

According to the PCR results for identification of virulence genes, 37 pathogenic strains were detected, of these 18 were ETEC (1.9%), 16 were EPEC (1.7%), and three were EIEC (0.3%). No strains belonging to the EHEC pathotype were recovered. Six ETEC strains (four from diarrhea cases and two from controls) show both LT and ST determinants. The sole presence of the gene for ST toxin was revealed in three strains and that of LT toxin in 9 strains. Nine out of 16 enteropathogenic *E. coli* were classified as atypical EPEC, since they lack the *bfpA* gene. All EPEC showed localized adhesion to HeLa cells, while atypical EPEC (*eae*<sup>+</sup>/*bfpA*<sup>–</sup>) showed a diffuse adherence pattern. Only three *ial* positive strains (EIEC) were found, all isolated among symptomatic children.

Adhesion tests on HeLa cells of the *E. coli* that tested negative for virulence genes in PCR, showed 67 aggregative patterns indicative of EAEC strains. Of these 50 (9.1%) were isolated from Group 1 and 17 (4.5%) from Group 2, with a statistically significant difference (*p* = 0.0067). A total of 167 strains, 125 (22.8%) from controls and 42 (11%) from cases, showed diffuse adhesion, indicative of DAEC strains (*p* < 0.0001) (Table 1(A)). All data have been also analyzed considering only *E. coli* strains isolated from children without other intestinal pathogens, as shown in Table 1(B). To better understand the effective correlation between EAEC and

Table 1

Frequency of occurrence of potentially pathogenic *E. coli* among children with (Group 1) and without (Group 2) diarrhea

	<i>E. coli</i> under study	ETEC	EPEC	EIEC	EAEC	DAEC	Cytotoxic <i>E. coli</i>	Diarrheagenic <i>E. coli</i>
<i>(A) Occurrence of pathotypes in stools</i>								
Group 1 ( <i>n</i> = 548)	375 (68.4%)	15 (2.7%)	13 (2.3%)	3 (0.5%)	50 (9.1%)	125 (22.8%)	23 (4.2%)	229 (41.8%)
Group 2 ( <i>n</i> = 380)	128 (33.7%)	3 (0.8%)	3 (0.8%)	0	17 (4.5%)	42 (11.0%)	4 (1.1%)	69 (18.1%)
Total ( <i>n</i> = 928)	503 (54.2%)	18 (1.9%)	16 (1.7%)	3 (0.3%)	67 (13.3%)	167 (33.2%)	27 (5.3%)	298 (32.1%)
<i>p</i> Value	<0.0001	0.0499	0.0768	–	0.0067	<0.0001	0.0048	<0.0001
<i>(B) Occurrence of E. coli pathotypes not associated with other pathogens (parasites and rotavirus)</i>								
Group 1 ( <i>n</i> = 548)		6 (1.1%)	8 (1.5%)	2 (0.4%)	26 (4.7%)	59 (10.8%)	9 (1.6%)	110 (20.0%)
Group 2 ( <i>n</i> = 380)		0	3 (0.8%)	0	6 (1.6%)	24 (6.3%)	2 (0.5%)	35 (9.2%)
Total ( <i>n</i> = 928)		6 (0.6%)	11 (1.2%)	2 (0.2%)	32 (3.4%)	83 (8.9%)	11 (1.2%)	145 (15.6%)
<i>p</i> Value		–	0.5397	–	0.0098	0.0195	0.2155	<0.0001

The *p* value was calculated according to Fisher exact test.

DAEC isolates with diarrhea, we analyzed their association with other pathogens (parasites and rotavirus) in stools. Regarding EAEC, 26 (4.7%) isolates from symptomatic and 6 (1.6%) isolates from asymptomatic children resulted not associated with other pathogens and this difference was highly significant ( $p = 0.0098$ ). Regarding DAEC, the importance of which as cause of enteritis is still disputed, the number was reduced to 59 (10.8%) in cases and to 24 (6.3%) in controls, the difference being still significant ( $p = 0.0195$ ).

Since cytotoxic *E. coli* might be regarded as a potential agent of enteric diseases, we considered in this study 27 *E. coli* isolates, that showed a supernatant mediated cytotoxicity on different cell lines as the unique pathogenic phenotype. The majority of cytotoxic *E. coli* (23 isolates) was found in feces from children with diarrhea, and only four in feces from the control group ( $p = 0.0048$ ). Analyzing the association of cytotoxic strains with other pathogens only nine *E. coli* from the group of cases, and two from controls were not found to be associated and the numbers were not significant.

#### 4. Discussion

The circulation of diarrheagenic *E. coli* has been investigated in this study. A total of 928 children aged less than seven years, living in a deprived zone of Maputo, in Mozambique, has been divided in two groups, on the basis of the presence/absence of gastrointestinal symptoms. *E. coli* have been isolated in a higher percentage of stools from children with diarrhea (68.4%), in comparison to asymptomatic ones (33.7%). It could be due, at least in part, to the modifications that occur in the intestinal environment during episodes of diarrhea, that can render it more favorable to *E. coli* colonization [14]. On the other hand, the presence of diarrheagenic *E. coli*, that can be per se responsible of symptoms, could explain this significant difference. In fact we found potentially diarrheagenic *E. coli* in 41.8% of symptomatic and in 18.1% of asymptomatic children.

PCR assays targeting virulence genes revealed a limited circulation of EPEC and ETEC, a rather rare presence of EIEC and no EHEC strains among 375 *E. coli* isolated from the children with diarrhea included in this study. Among the 18 enterotoxigenic *E. coli*, six possess genes for both ST and LT toxins, nine harbor only LT and three only ST genes, in agreement with the well-known variability pathogenic profile in ETEC strains [8]. Nine out of 16 EPEC isolates showed the absence of the *bfpA* virulence factor, correlated to the expression of bundle forming pilus. These *E. coli* show a diffuse rather than localized adhesion to target cells, in agreement with other results showing that the phenotype, known as atypical EPEC, might still be able to adhere to the intestinal mucosa and to cause diarrhea [8]. Atypical EPEC are now predominant in industrial countries, while typical EPEC are more frequently isolated in developing countries [15]. However, some recent reports indicate an increased isolation of atypical EPEC also in developing countries such as Brazil [15] and South Africa, where 28.2% of the strains isolated from children with diarrhea possess the *eae*, but not the *bfpA* gene [16]. In the present study atypical EPEC are about half of the total EPEC isolates, suggesting an epidemiological situation of transition in the survey area. The reason for this transition is not clear, but it may be related to changes in living conditions and feeding habits. In fact, typical EPEC are not found in animals, suggesting that humans are the only reservoir for this pathotype. On the contrary, most atypical pathotypes have been isolated from different animal species including cattle, rabbits, dogs and monkeys, thus supporting the reservoir hypothesis for animals that live in close association with humans or are used as food resources. It would be very useful to compare human and animal strains isolated in the area of the study by molecular epidemiology tools.

The absence of EHEC strains is not surprising since this pathotype is not frequently isolated in Southern Africa, although one of the largest outbreaks of O157 EHEC occurred in Swaziland in 1992 [17]. In addition,



in other parts of Africa and in non-epidemic settings, EHEC appears to be more frequent in adults rather than in children [18].

Only in the 15.6% of children examined (20.0% for the symptomatic and 9.2% for the asymptomatic group), *E. coli* was the only potentially pathogenic microorganism observed, while in the majority of subjects the bacterium was associated with one or more infective agents such as viruses (Rotavirus), protozoa (*Giardia intestinalis*, *Cryptosporidium* and *Entamoeba histolytica*) and helminthes (*Ascaris lumbricoides*, *Trichiuris trichiura* and *Strongyloides* spp.). The fact that both symptomatic and asymptomatic children were found to be highly infected by enteropathogens, in some cases with multiple presences, depicts the poor environmental hygiene of the area under study and the inhabitants' low income that cannot guarantee standard health conditions for children.

In general, the aggregative pattern of adhesion is a strong evidence of the EAEC pathotype. The presence of 67 strains (13.3% of total sample) showing aggregative phenotype on epithelial cells, indicates that the most frequent pathogenic *E. coli* present in the population under study is enteroaggregative. This is in agreement with worldwide evidence that EAEC is emerging as a significant enteric pathogen, responsible for acute and persistent diarrhea and concurrent cause of malnutrition and growth defects in children [19]. Several reports from Sub-Saharan Africa indicate that EAEC is endemic among children within communities [20–22]. In this study, the presence of EAEC is significantly associated to symptoms of enteritis; this significance is maintained in the isolates not associated with other pathogens, such as rotavirus and/or parasites. This finding indicates a prominent role of this pathotype in children with diarrhea, also in Mozambique.

Cytotoxicity is generally shown by EHEC pathotypes. Interestingly, we observed cell detachment in HeLa cultures and toxicity in VERO and CHO epithelial cells in isolates without correlation with any major pathogenic genotype. This could be due to the presence of secreted enterotoxins such as CNF (cytotoxin necrotic factor), CDT (cytotoxic distending toxin) [8], PET (plasmid encoded toxin) [23] and ClyA cytotoxin protein [24] or other unknown factors related to cell cytotoxicity. Further investigation is required in order to assess the pathogenicity of these strains and their potential role as cause of diarrhea in children.

The results of this study indicate that potentially pathogenic *E. coli* are widely present among the population of children studied. Apart from DAEC whose pathogenic role in diarrhea is still being questioned, EAEC is the predominant *E. coli* pathotype in circulation. This should be taken into consideration for health intervention purposes since EAEC can give debilitating long

term consequences particularly in vulnerable groups such as children and immuno-deficient individuals.

Identification is an important task also in an African setting and needs an integrated approach, including both phenotyping and genotyping for the characterization of potentially pathogenic *E. coli* in diarrhea. In addition, molecular epidemiology [25] and phylogrouping [26] could better clarify the spreading and circulation of community acquired, pathogenic *E. coli*. Therefore, to support enteric disease control programs in the developing countries, there is an urgent need for simpler and cheaper techniques for the study of diarrheagenic *E. coli*.

### Acknowledgements

We thanks Dr. Carolina Araujo and all the staff of the Xipamanine Health Center for assistance in patient selection and sample collection, and Dr. Emma Gori for her unconditioned support. This project was supported by grants from the University Program, General Directorate Cooperation and Development of MAE, Italy, Sardinia Regional Government and Italian FIRB 2000 fund.

### References

- [1] Kaper, J.B., Nataro, J.P. and Mobley, H.L.T. (2004) Pathogenic *Escherichia coli*. *Nat. Rev.* 2, 123–140.
- [2] Clarke, S.C. (2001) Diarrhoeagenic *Escherichia coli*—an emerging problem?. *Diag. Microbiol. Infect. Dis.* 41, 93–98.
- [3] Simango, C. and Dindiwe, J. (1987) The aetiology of diarrhoea in a farming community in Zimbabwe. *Trans. R. Soc. Trop. Med. Hyg.* 81, 552–553.
- [4] Nicoletti, M.F., Superti, C., Conti, A., Calconi, A. and Zagaglia, C. (1988) Virulence factors of lactose-negative *Escherichia coli* strains isolated from children with diarrhea in Somalia. *J. Clin. Microbiol.* 26, 524–529.
- [5] Agbodaze, D., Abrahams, C.A. and Arai, S. (1988) Enteropathogenic and enterotoxigenic *Escherichia coli* as aetiological factors of infantile diarrhoea in rural and urban Ghana. *Trans. R. Soc. Trop. Med. Hyg.* 82, 489–491.
- [6] Ako-Nai, A.K., Lamikanra, A., Ola, O. and Fadero, F.F. (1990) A study of the incidence of enterotoxigenic *Escherichia coli* (ETEC) secreting heat-labile toxin in two communities in southwestern Nigeria. *J. Trop. Med. Hyg.* 93, 116–118.
- [7] Calundungo, R., Maddau, G., Rappelli, P., Cappuccinelli, P., Leite, F., Francisco, M. and Colombo, M. (1994) *Escherichia coli* and enteritis in Angola. *Lancet* 344, 538–539.
- [8] Nataro, J.P. and Kaper, J.B. (1998) Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev.* 11, 142–201.
- [9] Bekal, S., Brousseau, R., Masson, L., Prefontaine, G., Fairbrother, J. and Harel, J. (2003) Rapid identification of *Escherichia coli* pathotypes by virulence gene detection with DNA microarrays. *J. Clin. Microbiol.* 41, 2113–2125.
- [10] Vidal, R., Vidal, M., Lagos, R., Levine, M. and Prado, V. (2004) Multiplex PCR for diagnosis of enteric infections associated with diarrheagenic *Escherichia coli*. *J. Clin. Microbiol.* 42, 1787–1789.
- [11] Rappelli, P., Maddau, G., Mannu, F., Colombo, M.M., Fiori, P.L. and Cappuccinelli, P. (2001) Development of a set of

- multiplex PCR assays for the simultaneous identification of enterotoxigenic, enteropathogenic, enterohemorrhagic and enteroinvasive *Escherichia coli*. *Microbiologica* 24, 79–85.
- [12] Okeke, I.N., Lamikanra, A., Czezuln, J., Dubovsky, F., Kaper, J.B. and Nataro, J.P. (2000) Heterogeneous virulence of enteroaggregative *Escherichia coli* strains isolated from children in Southwest Nigeria. *J. Infect. Dis.* 181, 252–260.
- [13] Gomes, T.A.T., Blake, P.A. and Trabulsi, L.R. (1989) Prevalence of *Escherichia coli* strains with localized, diffuse and aggregative adherence to HeLa cells in infants with diarrhea and matched controls. *J. Clin. Microbiol.* 27, 266–269.
- [14] Tazume, S., Ozawa, A., Yamamoto, T., Takahashi, Y., Takeshi, K., Saidi, S.M., Ichoroh, C.G. and Waiyaki, P.G. (1993) Ecological study on the intestinal bacteria flora of patients with diarrhea. *Clin. Infect. Dis.* 2, 77–82.
- [15] Trabulsi, L.R., Keller, R. and Tardelli Gomes, T.A. (2002) Typical and atypical enteropathogenic *Escherichia coli*. *Emerg. Infect. Dis.* 8, 508–513.
- [16] Galane, P.M. and Le Roux, M. (2001) Molecular epidemiology of *Escherichia coli* isolated from young South African children with diarrhoeal diseases. *J. Health Popul. Nutr.* 19, 31–38.
- [17] Isaacson, M., Canter, P.H., Effler, P., Arntzen, L., Bomans, P. and Heenan, R. (1993) Hemorrhagic colitis epidemic in Africa. *Lancet* 341, 961.
- [18] Okeke, I.N., Ojo, O., Lamikanra, A. and Kaper, J.B. (2003) Etiology of acute diarrhea in adults in south-western Nigeria. *J. Clin. Microbiol.* 41, 425–430.
- [19] Okeke, I.N. and Nataro, J.P. (2001) Enteroaggregative *Escherichia coli*. *Lancet Infect. Dis.* 1, 304–313.
- [20] Okeke, I.N., Lamikanra, A., Steinrück, H. and Kaper, J.B. (2000) Characterization of *Escherichia coli* strains from cases of childhood diarrhea in provincial southwestern Nigeria. *J. Clin. Microbiol.* 38, 7–12.
- [21] Vila, J., Vargas, M., Casals, C., Urassa, H., Mshinda, H., Schelleberg, D. and Gascon, J. (1999) Antimicrobial resistance of diarrheagenic *Escherichia coli* isolated from children under the age of 5 years from Ifakara, Tanzania. *Antimicrob. Agents Chemother.* 43, 3022–3024.
- [22] Sang, W.K., Oundo, J.O., Mwituria, J.K., Waiyaki, P.G., Yoh, M., Iida, T. and Honda, T. (1997) Multidrug-resistant enteroaggregative *Escherichia coli* associated with persistent diarrhea in Kenyan children. *Emerg. Infect. Dis.* 3, 373–374.
- [23] Sui, B.Q., Dutta, P.R. and Nataro, J.P. (2003) Intracellular expression of the plasmid-encoded toxin from enteroaggregative *Escherichia coli*. *Infect. Immun.* 71, 5364–5370.
- [24] Wai, S.N., Westermark, M., Oscarsson, J., Jass, J., Maier, E., Benz, R. and Uhlin, B.E. (2003) Characterization of dominantly negative mutant ClyA cytotoxin proteins in *Escherichia coli*. *J. Bacteriol.* 185, 5491–5499.
- [25] Horvath, R., Dendis, M., Schlegelova, J., Ruzicka, F. and Benedik, J. (2004) A combined AFLP-multiplex PCR assay for molecular typing of *Escherichia coli* strains using variable bacterial interspersed mosaic elements. *Epidemiol. Infect.* 132, 61–65.
- [26] Johnson, J.R. and O'Bryan, T.T. (2000) Improved repetitive-element PCR fingerprinting for resolving pathogenic and non-pathogenic phylogenetic groups within *Escherichia coli*. *Clin. Diagn. Lab. Immunol.* 7, 265–273.