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Virulence, serotype and phylogenetic groups of diarrhoeagenic *Escherichia coli* isolated during digestive infections in Abidjan, Côte d'Ivoire

Adjéhi Dadie[1]*, Nazaire Kouassi[2], Etienne Dako[3], Marcellin Dje[1] and Mireille Dosso[4]

[1]University of Abobo-Adjamé, Faculty of food sciences and technology, Laboratory of biotechnology and food microbiology, 02 BP 801 Abidjan 02, Côte d'Ivoire.
[2]National Center of Agricultural Research (CNRA), Central Laboratory of Biotechnology, 01 Bp 1740 CNRA, Abidjan, Côte d'Ivoire.
[3]University of Moncton, Laboratory of biotechnology and molecular biology, ESANEF, N.-B. E1A 3E9 Moncton, Canada.
[4]University of Cocody, department of Medical Science; Head of Pasteur Institute, 01 Bp 490 Abidjan 01, Côte d'Ivoire.

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The virulence, serotype and phylogenetic traits of diarrhoeagenic *Escherichia coli* were detected in 502 strains isolated during digestive infections. Molecular detection of the target virulence genes, *rfb* gene of operon O and phylogenetic grouping genes *Chua, yjaA* and TSPE4.C2 was performed. Prevalence of strains harbouring virulent genes was 7.8%. The virulent genes *eaeA, bfp, stx2, st1, lt, aggA, east1, ipaH, ial, cnf1* and *afa* were detected. EAEC (36%) and both EPEC and ATEC (25.6%) are the most detected pathovars (p<0.05). STEC (5.1%), NFEC (7.7) and DAEC (7.7) are less represented. Serogroups are overall diversified (89%), however, serogroups O157, O103 and O86, previously known to be associated with virulence were revealed. Most of the *E. coli* pathovars (53%) belonged to phylogenetic group A and in decreasing importance order, to D (23.5%), B1 (11.7%) and B2 (11.7%) groups. The study shows a diversified population of intestinal strains (84.6%), with a low phenotypic and phylogenetic link lower (p<0.05). Due to the great diversity of pathotypes, continuous monitoring should be implemented to identify risk factors and major pathways of contamination that help defining strategies to reduce infections associated with *E. coli*.

Key words: *Escherichia coli*, virulence gene, serogroup, phylogenetic group, diversity.

INTRODUCTION

Escherichia coli are known as a component of the intestinal microflora of humans and most homeothermic or warm-blooded animals (Bettelheim, 1997). However, strains that have acquired virulence factors are involved in digestive and extra-intestinal infections (Donnenberg, 2002). The epidemiological significance of virulent *E. coli* is well known and of public health concern (EFSA, 2011, 2012). The most recent outbreak occurred in Germany, and has spread in many countries of the European Union (Bielaszewska et al., 2011; Frank et al., 2011).

*Corresponding author. E-mail: thomasdadie@yahoo.fr. Tel: (225)01 87 97 17 /57 11 13 72. Fax: (225)20 30 43 00.

Abbreviations: EPEC, enteropathogenic *Escherichia coli*; **ATEC,** atypical *E. coli*; **STEC,** shiga-toxin producing *E. coli*; **EAEC,** enteroaggregative *E. coli*; **ETEC,** enterotoxigenic *E. coli*; **NFEC,** necrotizing factor producing *E. coli*; **DAEC,** diffusely adherent *E. coli*; **EIEC,** enteroinvasive *E. coli*.

This outbreak was linked to the emergence of unusual serotype O104:H4 of Shiga toxin-producing *E. coli* (STEC), although with genetic proprieties related to enteroaggregative *E. coli* (EAEC) (Rasko et al., 2011). The outbreak has made an impact of over 3332 STEC infections in patients, 818 haemolytic uremic syndrome (HUS) cases with nervous system complications and 36 deaths (Wieler et al., 2011). The alternative for the control of such infections is usually based on epidemiological surveillance, itself based on spatio-temporal documentation of phenotypic and molecular determinants of pathotypes (OMS/FAO, 2008). The production of this data reveals the diversity of strains, risk factors or the presence of infectious chains which constitute elements for the implementation of outbreaks prevention models. Phylogenetic characteristics play an important role for traceability and knowledge of the evolutionary history of pathovars (Chaudhuri and Henderson, 2012). Multilocus sequence typing (MLST) is considered as the gold standard for phylogenetic characterization (Goldberg et al., 2006). However, Clermont et al. (2000) have developed a method easier and faster, based on triplex PCR detection of *Chua* gene, involved in the transport of heme in *E. coli* O157:H7 (Bonacorsi et al., 2000), yjaA gene characterized in the genome of *E. coli* K-12 (Blattner et al., 1997) and the anonymous fragment TSPE4.C2 (Bonacorsi et al., 2000). Using this method, it was possible to determine the relationship between virulent clones of *E. coli* and their phylogenetic group (Clermont et al., 2000).

Data on the molecular characteristics of pathogenic *E. coli* are rare in Côte d'Ivoire, while their involvement in frequent infectious diarrhea is established (Guessennd et al., 2008; Dadie et al., 2000). Recent studies on the subject are limited to a few virulence determinants and did not take into account many of the ten listed pathovars (Dadie et al., 2010; 2000). In addition, it has not been mentioned in previous studies of phylogenetic characteristics of strains.

The objective of this study was to determine the diversity of *E. coli* strains isolated during diarrheas, based on virulence determinants, serogroups and phylogenetic groups.

MATERIALS AND METHODS

Strains and sampling

Study material consisted of *E. coli* strains isolated from stool during infectious diarrheas among children and adults patients. They were collected from 2009 to 2011 in the laboratories of bacteriology at the Pasteur Institute of Côte d'Ivoire, Centre Hospitalier Universitaire (CHU) of Yopougon, CHU of Treichville, and the National Institute of Public Health of Adjamé (INSP). Reference and control strains of *E. coli* were used to validate the tests. These are *E. coli* ATCC 35401, which harbored virulence genes *lt, st*; *E. coli* M19 (*stx1, stx2*) obtained from the University of Brno, Czechoslovakia; *E. coli* Ec-368 (*stx1, stx2*), B2F1 (saa), received from Pasteur Institute, Paris, France; *E. coli* ATCC 43887 (*eaeA, bfpA*); *E. coli* O157:H7 EDL 933 (Schmidt et al., 1995). The sampling included strains isolated during a waterborne poisoning, which occurred in March 2010 in the municipality of Attécoubé in Abidjan. A total of 502 strains of *E. coli* were collected. The distribution of strains collected was as follows: 104 strains from children younger than 5 years, 197 from young children aged from 5 to 14 years and 201 patients aged more than 14 years. The control of collected strains identity was carried out, using API 20E (BioMérieux, Marcy l'Etoile France). Among the strains analysed, 230 were kept for at least 6 months at -70°C before the detection of virulence factors.

Virulence genes detection

A 200 µl suspension of bacterial culture of 24 h was boiled for 10 min at 100°C and the 10^5 g centrifuged solution resulting of this thermal shock was used as template DNA for virulence genes detection. The primers used are shown in Table 1. The amplification reaction was performed by PCR in a 25 µl reaction mixture consisting of 10X buffer (Bio-Rad, Marnes-La-Coquette, France), 1.5 mM $MgCl_2$ (Bio-Rad, Marnes-La-Coquette, France), 200 µM of each deoxynucleotide triphosphate (dNTP) (Fischer-Canada), 20 pmol of each primer (Sigma-Aldrich, Canada Ltd), 5 U/µl of Taq DNA polymerase (Bio-Rad, Marnes-La-Coquette, France) and 5 µl of the template DNA. The amplification was performed in a thermal cycler, Perkin Elmer 9700 (Applied Biosystems, USA). The amplification program includes an initial denaturation at 94°C for 3 min; 30 cycles of 1 min denaturation at 94°C, 45 s annealing at 56°C and 1 min elongation at 72°C; followed by a final extension for 5 min at 72°C. The revelation of the amplification products was performed on agarose gel 1.5% with ethidium bromide, 0.5 mg / ml.

Molecular serotyping

DNA extraction and purification

The molecular serotyping was performed using a purified DNA extract, according to the protocol of the Promega®Kit, "Wizard Genomic DNA Purification Kit". The *E. coli* strain to be studied was cultured in a tryptic-casein-soy broth (BioMérieux, Marcy l'Etoile France) for 18 h at 37°C. From the obtained suspension culture, 1 ml was collected in a 1.5 ml Eppendorf tube and centrifuged at 13000 *g* for 2 min. The pellet obtained was taken up in 600 µl of "nuclei lysis" solution (Promega, USA) and cell lysis was performed for 5 min at 80°C. To this lysate was added 3 µl of RNAse solution (Promega, USA). After homogenization, the mixture was incubated for 1 h at 37°C and vortexed after the addition of 200 µl of "Protein Precipitation" solution (Promega, France). The mixture was incubated for 5 min in an ice bath and centrifuged at 13000 *g* for 3 min. The supernatant was mixed with 600 µl of isopropanol, homogenized and centrifuged at 13000 *g* for 2 min. The pellet was resuspended in 600 µl of 70% ethanol and centrifuged at 13000 *g* for 2 min. After aspiration of ethanol, the tube was air dried for 15 min and the pellet was dissolved in rehydration solution and stored at 4°C.

PCR-RLFP for detection of operon O rfb gene

Typing procedure was performed according to Coimbra et al. (2000) method, slightly modified. The reaction was carried out, using kit reagents "Expand Long Template PCR System" (Boehringer, Mannheim, Germany). The primers used, 412.5'-CAC TGC CAT ACC GAC GCC GAT CTG TTG CTT GG-3' and 412.5'-ATT GGT AGC TGT AAG CCA AGG GCG GTA GCG T-3', are respectively complementary to JUMPstart and *gnd* (Wang and Reeves, 1998). A

Table 1. Primers used for PCR and amplicon size expected.

Gene	Primer sequence	Molecular size (pb)	References Gen bank/EMBL, Number
stx1	stx1f-5'-GAAGAGTCCGTGGGATTACG-3' stx1r-5'-AGCGATGCAGCTATTAATAA-3'	130	AF461172
stx2	stx2-5'-ACCGTTTTTCAGATTTTACACATA-3' stx2-5'TACACAGGAGCAGTTTCAGACAGT-3'	298	AY143337
st1	fp: 5′-TTTCCCCTCTTTTAGTCAGTCAACTG- bp: 5′-GGCAGGATTACAACAAAGTTCACAG-3′	160	M25607
east1	East1F-5'- CCATCAACACAGTATATCCGA-3' East1R- 5'- GGTCGCGAGTGACGGCTTTGT-3'	111	Monteiro-Nato et al. (1997)
cnf1	CNF1F-5'- GCTCAACGAGACTATGCTCTG-3' CNF1R-5- ACGCTGCTAAGTACCTCCTGG-3'	278	Falbo et al. (1992)
eaeA	eae5'-CACACGAATAAACTGACTAAAATG-3' eae5'-AAAAACGCTGACCCGCACCTAAAT-3	376	AE005595
bfpA	bfp f- 5'-TTCTTGGTGCTTGCGTGTCTTTT-3' bfp r'-5'-TTTTGTTTGTTGTATCTTTGTAA-3'	367	Yatsuyanagi et al. (2002)
ipaH	ipaHf-5'- TGGAAAAACTCAGTGCCTCT-3' ipaHr-5'- CCAGTCCGTAAATTCATTCT-3'	423	Lüscher et Altwegg (1994)
ial	ial F -5'-CTGGATGGTATGGTGAGG-3' ial R'-5'-GGAGGCCAACAATTATTTCC-3'	320	Frankel et al. (1989)
aggA	aggA -5'- AGACTCTGGCGAAAGACTGTATC -3' aggA5' ATGGCTGTCTGTAATAGATGAGAAC-3'	194	Schmidt et al. (1995)
lt	LT.f-5'- TCTCTATGTGCATACGGAGC-3' LT.r-5'- CCATACTGATTGCCGCAAT-3'	322	Frankel et al. (1989)
afa	afa f- 5'- CAGCAAACTGATAACTCTC-3' afa r-5'-CAAGCTGTTTGTTCGTCCGCCG-3'	750	Le Bouguenec et al. (1992)

50 µl reaction mixture, was constituted with 30.25 µl of PCR water (Fischer-Canada), 2.5 µl of dNTP, 10 mM, 1.5 µl of each primer, 15 pM, 5 µl of 10 X buffer, 1 µl of Promega®kit extract of template DNA and 3.5 U/µl of Taq polymerase (Bio-Rad, Marnes-La-Coquette, France). The amplification program consisted of two phases. Phase 1 composed of initial denaturation at 94°C for 2 min; 10 cycles composed of denaturation at 94°C for 10 s, annealing at 63°C for 30 s and elongation 68°C for 15 min. Phase 2 consist in 20 cycles of denaturation at 94°C for 10 s, annealing at 63°C for 30 s, elongation at 68°C for 15 min, 20 s and final elongation at 72°C for 7 min. Amplification products were subjected to a restriction by *Mbo*II (Amersham-Pharmacia-Biotech), in a reaction mixture of 25 µl, composed of 21 µl of amplification products, 2.5 µl of 10 X buffer and 1.5 µl of *Mbo*II, 12 U/µl (endonuclease). The restriction was performed at 37°C for 3 h and an irreversible denaturation of *Mbo*II was carried out by thermic shock at 72°C for 10 min. The digestion products were run on 2% gel consisting of 1% standard agarose and 1% Metaphor agarose in TAE buffer 0.5 X. The molecular weight marker used was composed of five volumes Amplisize (Bio-Rad, Marnes-La-Coquette, France) and a volume of lambda *Hind*III (Promega, USA). The data was processed with the software RestrictoTyper®(Institut Pasteur, Paris, France) and molecular profiles were translated into O antigen.

Determination of phylogenetic groups

The method of Clermont et al. (2000) was adapted to the experimental conditions of this study, by changing in certain components concentration of the reaction mixture and the amplification parameters. This method is based on the realization of a triplex PCR, using characteristic primers of genes *Chua* (Whittam, 1996; Bonacorsi et al., 2000), *yjaA* (Blattner et al., 1997) and the anonymous fragment TSPE4.C2 (Bonacorsi et al., 2000). The sequences of the three primers pairs used are reported in Table 2. The 25 µl reaction mixture consists of 1.5 mM of each dNTP,

Table 2. Primers sequences used for determination of phylogenetic groups.

Gene	Primer sequence	Molecular Size (pb)	Reference
ChuA	ChuA.1 -5'-GACGAACCA ACGGTCAGGAT-3' ChuA.2 5'-TGCCGCCAGTACCAAAGACA-3'	279	Whittam (1996)
YjaA	YjaA.1- 5'-TGAAGTGTCAGGAGACGCTG-3' YjaA.2 5'-ATGGAGAATGCGTTCCTCAAC-3'	211	Bonacorsi et al. (2000)
TspE4C2	TspE4C2.1.5'-GAGTAATGTCGGGGCATTCA-3' TspE4C2.2 5'-CGCGCCAACAAAGTATTACG-39	152	Clermont et al. (2000)

Table 3. Isolation frequency of virulent *E. coli.*

Patients class of age (year)	*E. coli* strains isolated	Virulent *E. coli* detected	Frequence SV (%)
< 5 years	104	19	18.2
5 - 14 years	197	11	5.6
> 14 years	201	9	4.5
Total	502	39	7.8

SV: Strains harboring virulence gene.

$MgCl_2$, 25 mM, 20 pmol of each of the three primers pairs, 10 X buffer, Taq DNA polymerase and 5 µl of DNA extract. Gene amplification was performed using an initial denaturation at 94°C for 4 min, 30 cycles of denaturation at 94°C for 20 s, annealing at 59°C for 15 s and elongation at 72°C for 30 s; followed by a final elongation for 6 min at 72°C. Interpretation of the results for classify species respectively in phylogenetic groups B2, D, A and B1 was performed according to the diagram of Clermont et al. (2000).

Statistical analysis

Categorical variables were compared using the Chi-squared test and Ficher's exact test (Armitage and Berry, 1987). The clonal relationship was assessed using Ward's method (Ward, 1963), which allows us to estimate the aggregation distance, through the creation of a dendrogram.

RESULTS

Prevalence of virulents strains of *E. coli*

The search for virulence factors, performed on a total of 502 *E. coli* strains revealed 39 (7.8%) harboring virulence genes, of which 19 (18.2%) isolated from younger than 5 years children; 11 (5.6%) from young children of 5 to 14 years and 9 (4.5%) of persons aged more than 14 years (Table 3).

Pathovars and their virulence factors

Virulence genes corresponding to pathovars are presented in Table 4. Virulence factors *east1* and *aggA*, which are characteristic of EAEC pathovars, were the most frequently detected (38.5%). Virulence genes specific to EPEC or ATEC (*eaeA, bfp*) were also detected in 10 (29%) strains, among which 5 (12.8%) were typical EPEC and 5 (12.8%) atypical EPEC (ATEC). The results also showed the presence of *stx2* genes, *ial, ipaH, afa* and *cnf1* that are respectively some characteristic pathovars of STEC, EIEC, DAEC and NFEC.

In this study, eight of nine pathovars known as agents associated with diarrheas caused by pathogenic *E. coli* were detected. The EAEC pathovars were more frequently ($p<0.05$) isolated (36%), followed by typical or atypical EPEC (25.6%). STEC were rarely detected (5.1%). The majority of EPEC and almost all DAEC were isolated from the population of children under 5 years. However, EAEC were detected in the same proportions among both children and adults. The frequencies of isolates are respectively 2.7% for EAEC, 2.4% for typical and atypical EPEC and 0.6% for each pathovar EIEC, DAEC and NFEC.

Molecular profile of operon O *rfB* gene and serogroups

The electrophoresis of restriction products of the amplified gene rfb of the operon O is shown in Figure 1, for nine virulent strains. It shows a variety of profiles compared to the molecular weight marker at 6 strains. However, the profile of pathovar HE5 is apparently identical to that of pathovar Ha102. Pathovars HE10 and He80 also have a profile different from the previous, but apparently similar for both strains. Some profiles obtained (LC26, Lc71, Lf19) did not correspond to serogroup

Table 4. Frequency of pathovars and their virulence factors.

Pathovar	Frequency Nb (%)	Virulence gene	Frequency Nb (%)
EPEC and ATEC	10 (25.6)	*eae* *bfp*	9 (17.3) 6 (11.5)
STEC	2 (5.1)	*stx1* *stx2*	0 2 (4)
EAEC	14 (36)	*east1* *aggA*	12 (23.1) 8 (15.4)
ETEC	5 (13)	*st1* *lt*	2 (4) 3 (5.7)
NFEC	3 (7.7)	*cnf1*	3 (5.7)
DAEC	3 (7.7)	*afa*	3 (5.7)
EIEC	2 (5.1)	*ipaH* *ial*	2 (4) 2 (4)

Nb: Number; EPEC: enteropathogenic *E. coli*; ATEC: atypical *E. coli*; STEC: shiga-toxin producing *E. coli*; EAEC: enteroaggregative *E. coli*; ETEC: enterotoxigenic *E. coli*; NFEC: necrotizing factor producing *E. coli*; DAEC: diffusely adherent *E. coli*; EIEC: enteroinvasive *E. coli*:

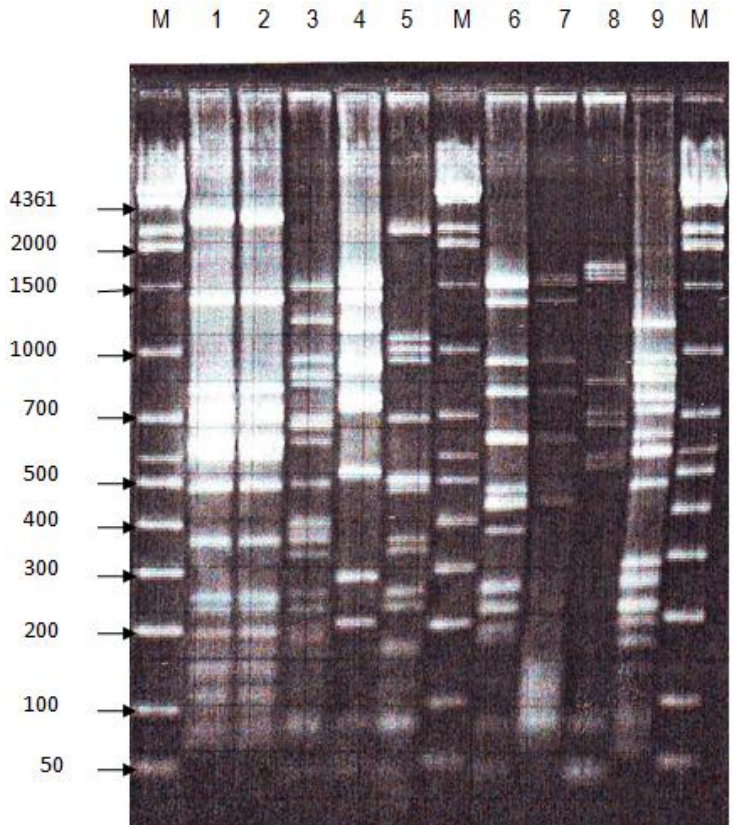

Figure 1. Electrophoresis of restriction products of the operon O *rfb* gene of *E. coli* strains. M: molecular weight marker, Pb: size in base pair; 1: He5 (O103); 2: Ha102 (O103); 3: He89 (O150); 4: Ha17 (O22); 5: He94 (O96); 6: He10 (O85); 7: He80 (O85); 8: He98 (O105); 9: Ha 87(O107).

Table 5. Pathovars distribution acording to their phylogenetic groups.

Phylogenetic group	EPEC	ATEC	ETEC	EIEC	STEC	EAEC	NFEC	DAEC	Total Nb (%)
A	2	1	4	1	0	7	1	2	18 (53)
B1.	0	2	0	0	0	1	0	1	4 (11.7)
D	2	0	0	0	2	4	0	0	8 (23.5)
B2	1	0	0	0	0	1	2	0	4 (11.7)
Total	5	3	4	1	2	13	3	3	34(100)

Nb: Number; EPEC: enteropathogenic *E. coli*; ATEC: atypical *E. coli*; STEC: shiga-toxin producing *E. coli*; EAEC: enteroaggregative *E. coli*; ETEC: enterotoxigenic *E. coli*; NFEC: necrotizing factor producing *E. coli*; DAEC: diffusely adherent *E. coli*; EIEC: enteroinvasive *E. coli*:

Table 6. Virulence, serotype and phylogenetic groups of *E. coli* strains**.**

Reference	Virulence	Sero group	Phylo group	Reference	Virulence	Sero type	Phylogroup
He2	*aggA*	O137	A	He83	*eae*		
He5	*cnf1*	O103	B2	He88	*aggA, east1*		A
He8	*afa*	O9	A	He89	*afa*	O150	A
He10	*eae, bfp*	O85	D	He94	*east1*	O96	A
He17	*lt*	O147	A	He96	*ipaH*		
He29	*east1, afa*		B1	He98	*east1*	O105	A
He33	*eae*	O109	A	He99	*aggA*		D
He45	*eae , bfp*	O43	A	He101	*eae, bfp*	O12	D
He78	*aggA, east1*	O130	D	He104	*ial*		A
He80	*eae, bfp*	O85	B2				
Ha11	*lt*	O110	A	Ha167	*stx2*		D
Ha17	*east1*	O22	D	Hd25	*lt*	O53	A
Ha31	*cnf1*	O39	A	Hd39	*aggA,*		B1
Ha41	*St1*	O141	A	Hd48	*aggA, east1*	O76	D
Ha46	*aggA, east1*		A	Hd79	*eae*	O80	B1
Ha55	*eae*	O86	B1	Hd91	*ipaH, ial*		
Ha68	*east1*		A	Hd108	*eae, stx2*	O157	D
Ha87	*bfp*	O107		Hd136	*east1*	O91	A
Ha102	*cnf1*	O103	B2	Hd140	*St1*	O17	A
Ha137	*eae, bfp*	O32	A	Hd177	*aggA, east1*	O40	B2

He: Strain from children <5 years, Ha: strains isolated from young children aged 5-14 years Hd: strains isolated from adults (> 14 years).

models in the database available after Taxotron treatment. The method used has revealed somatic antigen for 28 of 39 strains (72%). Strains HE5 and Ha102 belonged to the same O103 serogroup (Table 6). This was also the case of strains HE10 and He80 which belonged to serogroup O85. Serogroups where generally diverse and did not appear to be linked with age.

Electrophoretic profiles and phylogenetic groups of strains

The electrophoretic pattern of amplification products of genes obtained in our experimental conditions is shown in Figure 2. It shows bands of the four phylogenetic groups A, B1, B2 and D expected. It is a unique band of 279 base pairs (bp) of the *Chua* gene, indicating membership of strains HE10, HD48 and Ha17, to group D, the three bands of 279, 211 and 152 bp of the strain HE5, corresponding respectively to *Chua*, *YjaA* and TspE4C2X, which reflect their membership to group B2 strain; the 211 bp single band of strain Cg27 (group B1) and 152 bp single band (VH50) for belonging to the group A.

The phylogroup was revealed in 34 (87.2%) strains on a set of 39 isolated (Table 5). In descending importance order, the pathovars belonged to phylogenetic group A (53%), D (23.5%), B1 (11.7%) and B2 (11.7%). There was no pathotype belonging to a specific phylogenetic group. In addition, phylogenetic group membership was not also related to specific serotypes and strains ecosystem.

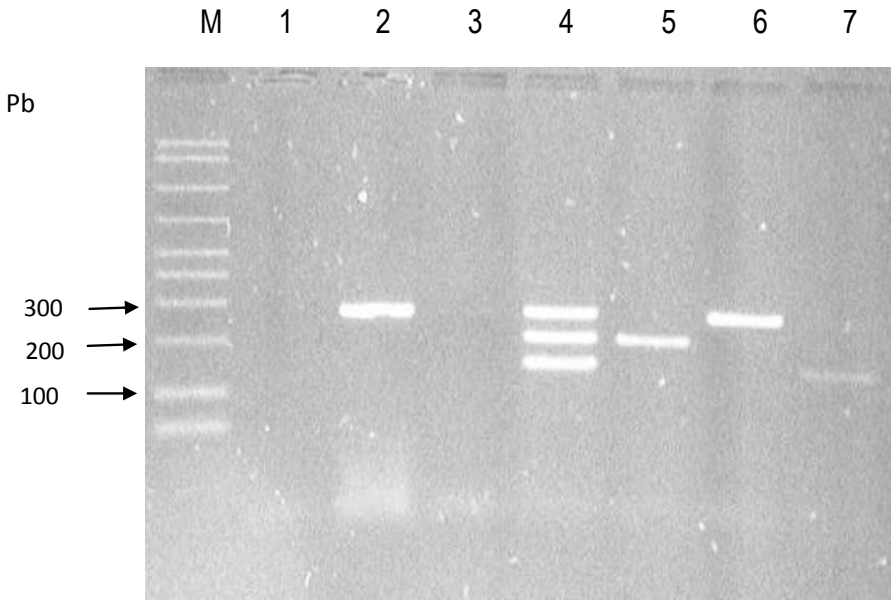

Figure 2. Electrophoretic pattern of amplification products of genes characteristic of phylogenetic groups. M: molecular weight marker, Pb: size in base pair; 1: He9; 2: He10; 3: Ha17; 4: He5; 5: Cg27; 6: Hd48; 7: Vh50

Table 6 shows the diversity on the distribution of pathovars in conventional phylogenetic groups. However, strains Ha102 and HE5, which shared the same virulence factor, as well as serotype, belonged to the same phylogenetic group (B2). As for pathovars HE10 and He80 which were bound by the virulence and serotype, they differed on their phylogenetic group. HE10 belongs to D and He80 to B2 group.

Clonal relation

The dendrogram of Figure 3 shows a low phylogenetic distance and thus a clonal link between pairs of species HE8 - He89 (A), HE10 - HE101 - He80 (D), He55-Hd79 (B1) and HE5 - Ha102 (B2). Only the last pair belongs to the same serotype and phylogroup.

DISCUSSION

E. coli pathovars and human diarrheas

The use of PCR revealed the diversity of strains on the basis of traits related to virulence. A total of 502 strains of *E. coli* were isolated, of which 39 (7.8%) harbored virulence factors associated with agents of infectious diarrhea. The prevalence of virulent strains is similar to that (7.3%) obtained by Rappelli et al. (2005), in a study of agents of childhood diarrhea in Mozambique. However, similar studies, conducted in children less than five years in South Africa (Galane and Leroux, 2001) and Tunisia (Al-Gallas et al., 2007) gave respectively 32.6 and 65%. The low prevalence of virulent *E. coli* in humans obtained in this study may be due to the fact that sampling took into account the strains isolated during a waterborne poisoning, occurred in March 2010 in the municipality of Attécoubé in Abidjan. A set of 170 strains of *E. coli* were isolated, of which only 5 (3%) had virulence factors. In addition, the fact that several strains were stored at -70°C for at least six months before analyses could lead to a loss of virulence factors (Markoulatos et al., 2002) and therefore also have an impact on reducing overall prevalence of virulent strains.

The results of our study show that almost half of the virulent strains (49%) were isolated from children less than 5 years. This result confirms those reported in previous work (Okeke et al., 2003; Valentiner-Branth, 2003; Rappelli et al., 2005), namely that children of this age group, represent the population most exposed and vulnerable to diarrhea of virulent *E. coli*. Among the 39 virulent strains of *E. coli*, 14 (36%) were entero-aggregative *E. coli* (EAEC). The EAEC pathovars would therefore be most frequently involved in human diarrhea in our environment. This observation not only confirms our previous results (Dadie et al., 2000), but also shows the emerging nature of this pathovar, as reported by other authors (Veilleux and Dubreuil, 2005; Franck et al., 2011). It is also known that EAEC play a special role in chronic diarrhea in people with HIV/AIDS infection (Kelly et al., 2003; Gassama-Sow et al., 2004). The high proportion of these pathovars in human strains studied may be due to the fact that several strains collected during sampling came from CHU of Treichville (Abidjan),

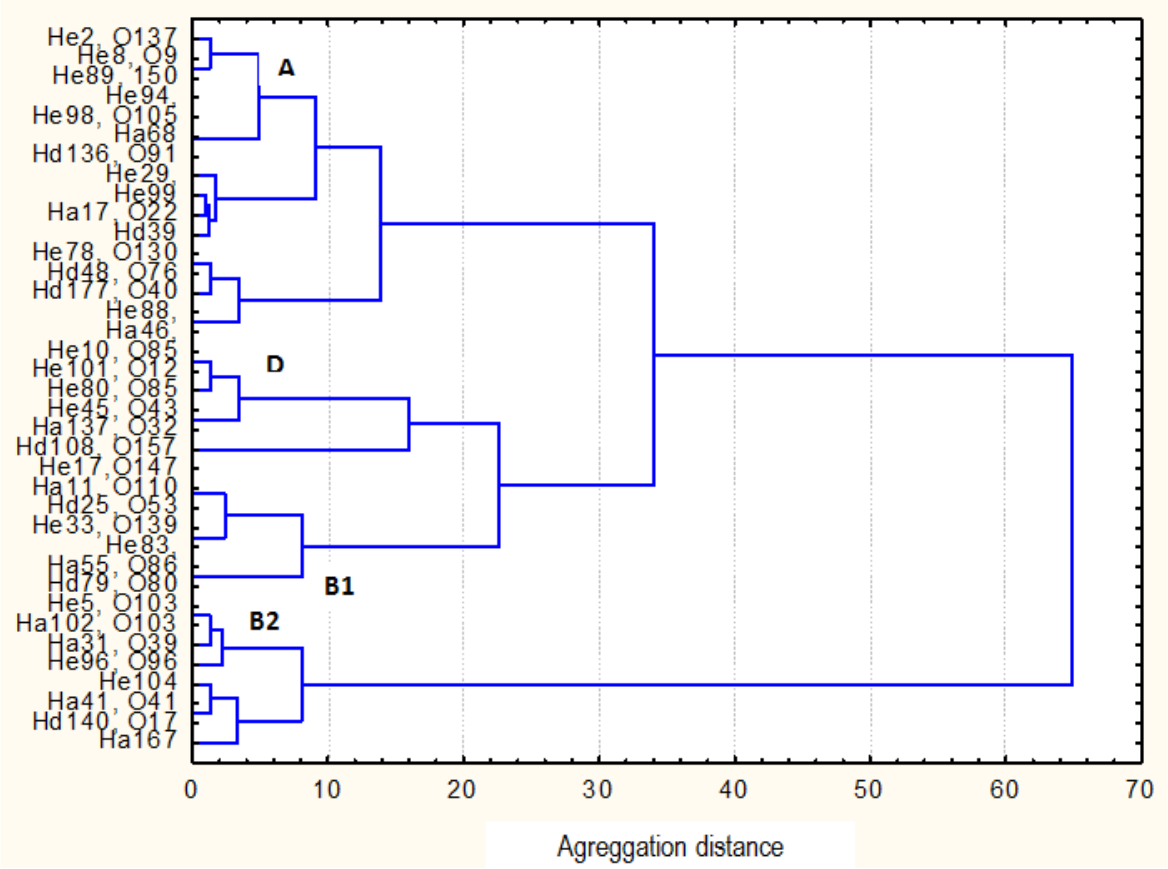

Figure 3. Dendrogram showing the phenotypic and phylogenetic relationship between pathovars of *E. coli* of human origin.

in a service receiving preferentially seropositive patients for HIV/AIDS. In this study, the rate of EPEC was 25.6 and in 60% of cases strains came from children under 5 years old. Many studies of EPEC on this part of the population showed that these pathovars were the leading cause of diarrhea in infants and children less than five years in Nigeria (Okeke et al., 2000), India (Bhan et al., 1989) and Bangladesh (Albert et al., 1991). Classification based on virulence determinants distinguishes the typical EPEC (*eae, bfp*) from atypical EPEC or ATEC (*eae*) which lacks EAF plasmid, encoding the *bfp* gene (Nataro et Kaper, 1998; Tobe et al., 1999).

In this study, on all 10 EPEC suspected, 5 (50%) presented characteristics of typical EPEC and 5 others, those of ATEC. The results show that ATEC are common in our environment and are involved in diarrheas like ECEP. Data confirms those of some authors (Nguyen et al., 2005; Orlandi et al. (2006), showing that the ATEC are widespread and sometimes dominant in many developing countries, but others found rather a predominance of typical EPEC on ATEC (Sooka et al., 2004: Moyo et al., 2007). Moreover, Nataro and Kaper (1998) indicated the difficulty of detecting specific *bfp* gene. The consequence is that the rate of EPEC detected in humans, could be better than the reality.

The ETEC pathovars represented 13% of the strains isolated during the infectious diarrhea associated with virulent *E. coli* in this study. The rates obtained by Okeke et al. (2003) in Nigeria and Sooka et al. (2004) in South Africa, respectively, were 8.8 and 8.42%. A greater importance of ETEC (32.4%) was found in Tunisia during the study of Al-Gallas et al. (2007). Each pathovar EIEC, DAEC and NFEC was detected, respectively, at a rate of 5.1 and 7.7%. The revelation of EIEC was rare or impossible in the studies respectively carried out in Guinea-Bissau (Valentiner-Branth et al., 2003), Mozambique (Rappelli et al., 2005), South Africa (Sooka et al., 2004) and Nigeria (Okeke et al., 2000; 2003). However, during a study on human diarrheas in Tunisia, EIEC pathovars were isolated by Al-Gallas et al. (2007) with a prevalence of 11.3%. The DAEC were all isolated from children under 5 years. Giron et al. (1991) reported that these pathovars were involved in diarrhea occurring most frequently in children less than five years, according to a mechanism of cell invasion similar to EPEC. However, some authors (Gunzberg et al., 1993; Nataro and Kaper, 1998) showed that DAEC did not always induce diarrhea in children. According to them, children

and adults are carriers of this type of pathovars, without evidence of specific symptoms. In addition, *E. coli* is known as the first agent of urinary tract infections (Stamm, 2002) in which the DAEC and NFEC pathovars are mostly involved (Santos et al., 2006), so the significance of the isolation of this type of pathovars in infectious diarrhea remains questionable.

Two strains of STEC harboring the *stx2* gene were isolated. The study confirms in this regard that STEC are not important etiological agents of diarrhea associated with *E. coli* in our environment, as we previously reported (Dadié et al., 2000). However, due to the evolving of epidemiological role of STEC in several African countries (Isaacson et al., 1993; Koyange et al., 2004; Hiko et al., 2008, Al-Gallas et al., 2007; Okeke et al., 2003), the establishment of a monitoring mechanism is necessary.

Serogroups and phylogenetic groups

The results of molecular serogrouping by detecting the *rfb* gene of operonO, do not generally show significant relationships between serotypes and pathotypes, but show a diversity of serogroups. Identified serogroups, including O157, O103 and O86 are known to be related respectively to pathovars STEC (O157 and O103) (Coimbra et al., 2000) and EPEC (O86) (Donnenberg, 2005; Kaper, 2004). If in the course of this study, the virulence genes classically attributed to these pathotypes were detected for some (O157 and O86); it is not for serogroup O103 which was rather carrying the gene *cnf1* instead of Shiga-toxin. The serogroup O86 was also not systematic to EPEC class, but may be characteristic of ETEC according to Germani (1995).

The multilocus sequence typing (MLST) is considered as the gold standard for phylogenetic grouping of species (Goldberg et al., 2006). However, the application of the method of Clermont et al. (2000) on our strains gave a sensitivity of 87% and a diverse phylogenetic distribution, with the majority (53%) of pathovars belonging to group A. The pathovars belonging to B2 group accounted for 11.7% and group D, 23.5%. During a study on 64 clinical strains, most of which composed of extra intestinal species, Clermont et al. (2000) found a distribution of 58% of B2 group agents and 17.1% of group D. It is established that most extra intestinal pathogenic strains belong to group B2 and to a lesser extent to group D (Bonacorsi et al., 2000). The low pathovars belonging to group B2 obtained in our study may be justified by the fact that the strains studied were of intestinal origin.

Both STEC (stx2) isolated in this study belonged to phylogenetic group D. The phylogenetic grouping of 10 strains of *E. coli* O157: H7, carried by Clermont et al. (2000) also showed that all isolates belonged to group D. Overall, the intestinal strains of our environment belonged to diverse phylogenetic groups, in contrast to what is known of extraintestinal strains that have a clonal population more than homogeneous (Clermont et al., 2000).

Clonal relationship between pathovars

The pathovars of the human ecosystem resulting from our study, were characterized by a high (89%) diversified population. The clonal relation is not significant between pathovars and there is no chain of contamination, may be due to one or some specific pathotypes. However, the markers of diversity used, show similar characteristics for two NFEC pathovars belonging to phylogenetic group B2. We know that the majority of extra intestinal pathovars belong to group B2 (Bonacorsi et al., 2000) and that NFEC also are frequently involved in urinary tract infections (Bielaszewska, 2007). The NFEC O103 clone detected could be a strain originally extra intestinal, probably an agent of urinary tract infection that transiently contaminated the digestive tract to be isolated during infectious diarrhea. In our study, two EPEC with similar phenotypic and genotypic factors were also isolated. It could be a single clone associated to infantile diarrhea and circulating in our environment. But to support this assertion, this clone should be isolated more frequently and an additional differentiation of strains of pulsed field gel electrophoresis (PFGE) type should be performed.

Conclusion

The study of strains diversity based on their virulence traits revealed the eight pathovars sought with greater frequency of EAEC and EPEC. The rare STEC highlighting should be seen as a warning, given to the epidemiological significance of these pathovars in several countries. Molecular serotyping performed by detecting the *rfb* gene of the operon O shows a variety of serogroups. Some serogroups conventionally known to have a link with specific pathovars were however identified. The majority of pathovars belongs to phylogenetic group A, although the phylogenetic group B2 agents have been highlighted. Belonging to group B2 is not specific for particular pathotypes. The study shows overall a relatively weak link between pathotypes. The great diversity of pathovars requires surveillance of virulent *E. coli*, for successfully identifying risk factors and the major routes of contamination, which determines the control of infections associated with pathovars.

Conflict of interests

The author(s) have not declared any conflict of interests.

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