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DETECTION OF THE VIRULENCE GENES OF DIARRHEAGENIC *E. COLI* BY 16-PLEX PCR FROM CHILDREN WITH DIARRHOEA IN OUAGADOUGOU, BURKINA FASO

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ABSTRACT
Diarrheal diseases are an important public health and economic problem worldwide, especially in Burkina Faso. This prospective study aims to determine the prevalence of major diarrheagenic *Escherichia coli* (DEC) pathotypes in stool samples from patients suffering from diarrheal diseases in four hospitals in Ouagadougou. After obtaining the informed consent, a total of 415 stool samples were collected from patients and *Escherichia coli* strains were identified using standard microbiological methods. We have used 16-plex PCR, which permit the identification of five different categories of diarrheagenic *E. coli* such as enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), shigatoxigenic *E. coli* (STEC), enteropathogenic *E. coli* (EPEC), and enterotoxigenic *E. coli* (ETEC) in a single reaction simultaneously. From the 292 strains, 23 (8%) were positive for DEC virulence genes with 12 (4%) samples being positive for EAEC, 5 (2%) for EPEC, 3 (1%) for EHEC, 2 (< 1%) for EIEC and 1 (< 1%) for ETEC. The children under 5 years old were most affected (74%). However, EAEC is the predominant DEC isolated from the examined patients with diarrhoea. This study indicated that the five major DEC pathotypes continuous to be a principal cause of diarrhoea in Burkina Faso. The present results will contribute to highlight the need of a surveillance program to reduce DEC prevalence in hospitals in this country.

KEYWORDS: Diarrheagenic *Escherichia coli,* 16-plex PCR, diarrhea, patients.

INTRODUCTION
Diarrheal disease is one of the most common causes of morbidity and mortality among children especially in developing countries (Al-Ghamdi *et al*., 2009; Alayed *et al*., 2013). The causes of diarrhoea include a wide range of viruses, bacteria, and parasites; among the bacterial pathogens, *Escherichia coli* play an important role (Nataro and Kaper, 1998).

There are diarrheagenic and non-diarrheagenic *E. coli* among *E. coli* isolates from stool, and it is difficult to distinguee them by colony morphology or biochemical tests (Kalnauwakul *et al*., 2007). The use of multiplex PCR methodology, which depends on detection of virulence genes, has provided a practical and rapid way of detecting diarrheagenic *E. coli* (DEC).

The diarrheagenic *E. coli* can be divided into six main type on the basis of distinct epidemiological and clinical features, specific virulence determinants, and association with certain serotypes: enteropathogenic *E. coli* (EPEC); enteroinvasive *E. coli* (EIEC); enterohemorrhagic *E.coli* (EHEC); enterohemorrhagic *E.coli* (EHEC); enterotoxigenic *E. coli* (ETEC); diffusely adherent *E. coli* (DAEC) (Hegde *et al*, 2012; Croxen *et al*., 2013). Infections of these pathogens strategies are associated to the presence of virulence genes and cell adhesion differ from one group to another (Nataro and Kaper, 1998).

Epidemiology of DEC in Africa is poorly understood due to the lack of facilities needed to characterize them. In Burkina Faso, previous studies revealed an important prevalence of DEC among children less than five years of old (Bonkoungou *et al*., 2013; Dembélé *et al*., 2015) but up to date no information is available on the prevalence of DEC in patient over five years old. In this study, we use a 16-plex-PCR (Antikainen *et al*., 2009) to detect simultaneously the five main pathotypes of *E. coli*

(EPEC, STEC, EAEC, ETEC and EIEC) from stool samples of patients under and over five year and adults.

MATERIALS AND METHODS

Study design and target population

This study was conducted between August and November 2014 in four different hospitals in Ouagadougou, the capital city of Burkina Faso (Fig 1). Study population was composed of children, young and old people with acute diarrhoea and who were hospitalized or received as external consultation in the health centre.

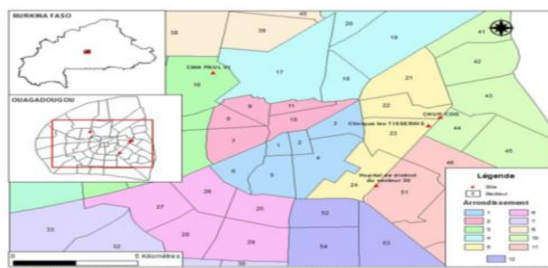

Fig 1: Map of Ouagadougou with the sites where sampling was done (CMA Paul VI, CHUP-CDG, Clinique les Tisserins and Hôpital du district de Bogodogo).

Stool samples

From August to November 2014, 292 stool samples were collected in sterile containers from diarrheal patients in 4 hospitals (CMA Paul VI, Centre Hospitalier Universitaire Pédiatrique Charles De Gaulle, Clinique les Tisserins and Hôpital du district de Bogodogo) in Ouagadougou and transported to the laboratory within 24 h in a cool box at 4 °C for immediate analysis. Sampling was done after obtaining the informed consent of patients.

Bacterial isolation

Isolation of suspected *E. coli* was carried out on to Eosin Methylene Blue (Liofilchem, Italy) and plates were incubated at 37°C for 18-24 h. The colonies with green metallic colour (suspect colonies) were selected and used for biochemical tests such as lactose, beta-glucuronidase, indol, citrate, and mannitol. The *E. coli* strains isolated were confirmed by using API 20E tests (BioMérieux, France).

Multiplex Polymerase Chain Reaction (16-plex PCR)

We used 16-plex PCR developed by Antikainen *et al.*, 2009 to detect the presence of STEC, EPEC, ETEC, EIEC and EAEC on stool samples. This PCR permit to detect simultaneously the following 16 genes uidA, pic, bfp, invE, hlyA, elt, ent, escV, eaeA, ipaH, aggR, stx1, stx2, estIa, estIb and ast. The primers and PCR conditions were as previously described (Antikainen *et al.*, 2009). The nucleotide sequences and predicted sizes of the amplified products for the specific oligonucleotide primers used in this study are shown in Table 1. The following criteria for identification of *E. coli* pathogroups were used: for STEC, the presence of stx1and/or stx2 and possibly eaeA, escV, ent and EHEC-hly; for EPEC the presence of eaeA and possibly escV, ent and bfpB (the absence of bfpB indicated aEPEC); for ETEC, the presence of elt and/or estIa or estIb; for EIEC,the presence of invE and ipaH; for EAEC, the presence of pic and/or aggR.

The confirmed colonies were used for DNA extraction. DNA extraction was performed using heating method (Moyo *et al.*, 2007). A loopful of bacterial growth of Mueller Hinton agar plate was suspended in 1 mL of sterile water. The mixture was boiled for 10 min à 100 °C and centrifuged for 10 min at 12000 rpm at 4 °C. The supernatant was collected and used in the PCR reactions. One (2.5) µl of supernatant was added to 22.5µl reaction mixture containing 5U of Taq DNA polymerase (AccuPower, korea) deoxyribonucleic triphosphate (10 mM), buffer GC (10 X), MgCl2 (25 mM), and PCR primers (escV, bfpB, stx1, stx2, LT, STIa, STI, invE, astA, aggR, pic, uidA, hly, eaeA, ipaH, ent) (10 µM) (STEC, STEC-ETEC, EAEC, EIEC, ETEC). Thermocycling conditions were as follows: 5 min at 94°C, followed by 30 amplification cycles of 94°C for 30s, 63°C for 60s and 72°C for 60s with a final extension of 72°C for 7 min on a thermal cycler (AB Appliee Biosystems). The PCR products were separated by electrophoresis in (1.5% weight/volume) agarose gel, stained with Redsafe solution (Prolabo, France) and visualized under UV light (Gel Logic 200). The following references strains were used in each PCR reaction: E2348-69 with the following genes: stx2, eae, escV, ent, EHEC-hly is define as EHEC or STEC, 17.2 with the following genes: aggR, pic, astA, uidA is define as EAEC, H907 with the following genes: invE, ipaH, uidA is define as EIEC and EDL 933 with the following genes: uidA, ent, escV, eae is define as EPEC.

Statistical analysis

The chi-square (χ^2) test or Fisher's exact test of MedCalc was used to determine the statistical significance of the data. A value of $p < 0.05$ indicated statistical significance.

RESULTS

Twenty tree DEC (8%) were detected out of the 292 samples, with 12 (4%) being positive for virulence genes of EAEC, 5 (2%) for EPEC, 3 (1%) for EHEC, 2 (< 1%) for EIEC and 1 (< 1%) for ETEC (Table 2). Among the different pathotypes EAEC, EPEC and EHEC virulence genes were most prevalent. Of the five EPEC isolates, four were atypical EPEC (aEPEC) and possessed only the gene *escV*, one EPEC was typical EPEC (tEPEC) and possessed only *bfp* gene. EHEC possessed the *EHEC-hly* gene and EIEC the gene *ipaH*. The one ETEC obtained possessed both genes *elt* and *estIa*. The prevalence of DEC was high (64.71%) among children less than five years old ($p < 0.0001$), with EAEC being the most prevalent in young children (Table 3). EHEC was only detected among children less than 1 year old, EIEC in children of 2 years old. However, the one ETEC was detected in children over 2 years old. According to the sex, DEC was significantly associated with male patients with diarrhoea ($p= 0.0357$).

Table 1: 16-plex PCR primers and the virulence genes detected.

Pathotypes	Target gene	Primer sequence (5′ to 3′)	Product size (bp)	Concentration (µM)	References
STEC, EPEC	*eaeA*	eae-F: TCAATGCAGTTCCGTTATCAGTT	482	0.1	37
		eae-R: GTAAAGTCCGTTACCCCAACCTG		0.1	
	escV	MP3-escV-F: ATTCTGGCTCTCTTCTTCTTTATGGCTG	544	0.4	39
		MP3-escV-R: CGTCCCCTTTTACAAACTTCATCGC		0.4	
	ent	ent-F: TGGGCTAAAAGAAGACACACTG	629	0.4	39
		ent-R: CAAGCATCCTGATTATCTCACC		0.4	
Typical EPEC	*bfpB*	MP3-bfpB-F: GACACCTCATTGCTGAAGTCG	910	0.1	39
		MP3-bfpB-R:CCAGAACACCTCCGTTATGC		0.1	
STEC	EHEC-*hly*	hlyEHEC-F: TTCTGGGAAACAGTGACGCACATA	688	0.1	12
		hlyEHEC-R: TCACCGATCTTCTCATCCCAATG		0.1	
	stx1	MP4-stx1A-F: CGATGTTACGGTTTGTTACTGTGACAGC	244	0.2	39
		MP4-stx1A-R: AATGCCACGCTTCCCAGAATTG		0.2	
	stx2	MP3-stx2A-F:GTTTTGACCATCTTCGTCTGATTATTGAG	324	0.4	39
		MP3-stx2A- R: AGCGTAAGGCTTCTGCTGTGAC		0.4	
EIEC	*ipaH*	ipaH-F: GAAAACCCTCCTGGTCCATCAGG	437	0.1	12
		ipaH-R: GCCGGTCAGCCACCCTCTGAGAGTAC		0.1	38
	invE	MP2-invE-F: CGATAGATGGCGAGAAATTATATCCCG	766	0.2	39
		MP2-invE-R: CGATCAAGAATCCCTAACAGAAGAATCAC		0.2	
EAEC	*aggR*	MP2-aggR-F: ACGCAGAGTTGCCTGATAAAG	400	0.2	39
		MP2-aggR-R: AATACAGAATCGTCAGCATCAGC		0.2	
	pic	MP2-pic-F: AGCCGTTTCCGCAGAAGCC	1,111	0.2	39
		MP2-pic-R: AAATGTCAGTGAACCGACGATTGG		0.2	
	astA	MP2-astA-F: TGCCATCAACACAGTATATCCG	102	0.4	39
		MP2-astA-R: ACGGCTTTGTAGTCCTTCCAT		0.4	
ETEC	*elt*	MP2-LT-F: GAACAGGAGGTTTCTGCGTTAGGTG	655	0.1	39
		MP2-LT-R: CTTTCAATGGCTTTTTTTTGGGAGTC		0.1	
	estIa	MP4-STIa-F:CCTCTTTTAGYCAGACARCTGAATCASTTG	157	0.4	39
		MP4-STIa-R: CAGGCAGGATTACAACAAAGTTCACAG		0.4	
	estIb	MP2-STI-F: TGTCTTTTTCACCTTTCGCTC	171	0.2	39
		MP2-STI-R: CGGTACAAGCAGGATTACAACAC		0.2	
E. coli	*uidA*	MP2-uidA-F: ATGCCAGTCCAGCGTTTTTGC	1,487	0.2	39
		MP2-uidA-R: AAAGTGTGGGTCAATAATCAGGAAGTG		0.2	

EAEC = Enteroaggregative *E. coli*, EPEC = Enteropathogenic *E. coli*, EIEC = Enteroinvasive *E. coli*, EHEC = Enterohemorrhagic *E. coli*, ETEC = Enterotoxigenic *E. coli*, µM = Micromolaire, [C] = Concentration, pb = "paire de base".

Table 2: Prevalence of diarrheagenic *E. coli* pathotypes.

	Virulence genes															
Pathovars	***uidA***	***pic***	***bfp***	***invE***	***EHEC-hly***	***elt***	***ent***	***escV***	***eae***	***ipaH***	***aggR***	***stx1***	***stx2***	***estla***	***estlb***	***astA***
Control strains																
17.2 (EAEC)	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+
EDL 933 (EPEC)	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-
H 907 (EIEC)	+	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-
E 2348-69 (EHEC)	+	-	-	-	+	-	+	+	+	-	-	+	+	-	-	-
Negative control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EHEC	-	-	-	-	3	-	-	-	-	-	-	-	-	-	-	-
Typical EPEC	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
Atypical EPEC	-	-	-	-	-	-	-	4	-	-	-	-	-	-	-	-
EIEC	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-
EAEC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12
ETEC	-	-	-	-	-	1	-	-	-	-	-	-	-	1	-	-

Table 3: Prevalence of DEC pathotypes according to the age and the sex.

Age (year) **Pathotypes**	0 – 5	6 - 10	11 - 15	16 - 20	+ 20	**Sex**		**Total**
						F	**M**	
EPEC	5	0	0	0	0	4	1	**5**
ETEC	1	0	0	0	0	0	1	**1**
EAEC	6	3	1	0	2	5	7	**12**
EIEC	2	0	0	0	0	0	2	**2**
EHEC	3	0	0	0	0	0	3	**3**
Total	**17**	**3**	**1**	**0**	**2**	**9**	**14**	**23**

Legend: M= male; **F**= female

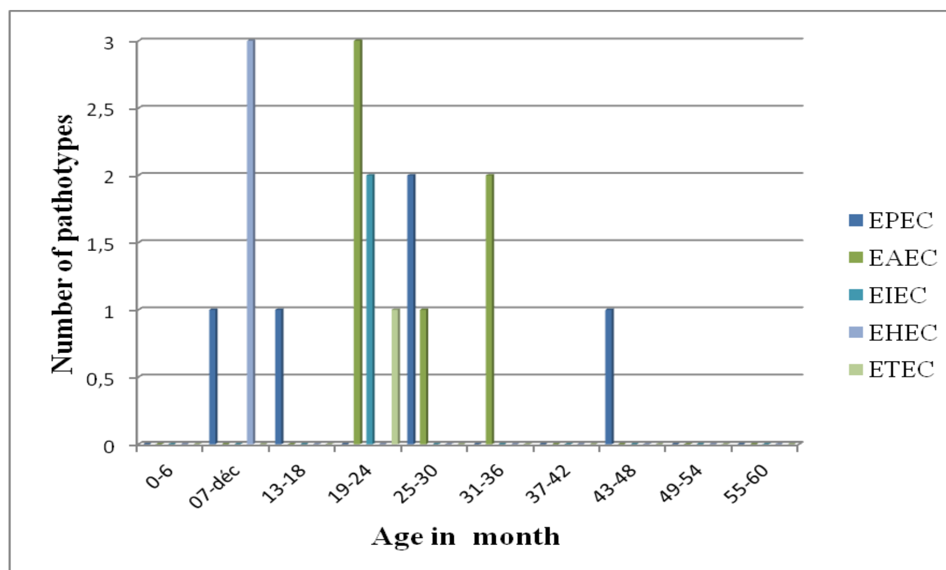

Fig 2: Distribution of DEC pathotypes among children under 5 years old.

DISCUSSION

In this study, we investigated the prevalence of the major diarrheagenic *Escherichia coli* (DEC) pathotypes by 16-plex PCR in 292 strains from patients with diarrhoea in four different health centres in Ouagadougou, Burkina Faso. Of the 292 strains tested, 23 (8%) were positive for DEC. Similar results have also been reported in Libya (Ali *et al.*, 2012). In contrast, higher prevalence of DEC was reported in Burkina Faso (Bonkoungou *et al.*, 2012) and in India (Priya *et al.*, 2010). This difference on the prevalence of DEC can be explained by the method used to isolate DEC strains (phenotypic assays) and also the PCR conditions (Brandal *et al.*, 2007).

In our study, EAEC was the most commonly detected DEC pathotypes, especially among children under five years old. Similar results have been reported by several authors, who concluded that EAEC was a predominant cause of persistent diarrhoea in developing country (Chaudhuri *et al.*, 2010; Hegde *et al*, 2012). The association of EAEC with diarrhoea appears to vary geographically, and many studies have demonstrated the importance of EAEC in pediatric diarrhoea (Priya *et al*, 2010; Rahouma *et al.*, 2011, Bonkoungou *et al.*, 2012). This fact could be explained by the lack of epidemiological characteristics of EAEC (likely sources, reservoirs of infection, routes of transmission, and seasonality).

EPEC are responsible for gastroenteritis in infants in developing countries. They are classified into two types based on the presence of the plasmid *E. coli* adherence factor (EAF). Typical EPEC (tEPEC) or type I is well recognized as a cause of gastroenteritis in infants (Hegde *et al*, 2012). They are associated with a higher risk of mortality in infants 0-11 months of age with moderate-to-severe diarrhoea (Lanata *et al*, 2013; Kotloff *et al.*, 2013). In this study, the typical EPEC was positive for *bfp* gene. These *E. coli* strains may carry virulence factors that may result in diarrheal disease by a perhaps unique mechanism of pathogenesis no yet identified. Indeed, Croxen *et al.* (2013) reported that cases of diarrhoea due to type I of EPEC decrease with age. This apparent resistance of adults and older children would be linked to the loss of receptors specific of typical EPEC with age or development of immunity (Nataro and Kaper, 1998). Ninety nine percent (99%) of EPEC find in this study was aEPEC. Similar results have been reported by Langendorf *et al.* (2015); Alikhani *et al.*, (2006); Trabulsi *et al.*, (2002). In addition, many authors have reported that aEPEC is more prevalent than tEPEC in both developed and developing countries, which is in concordance to our present results (Hernandes *et al*, 2009; Bonkoungou *et al*, 2012; Lozer *et al*, 2013; Dutta *et al.*, 2013). EHEC were detected in 3 (13%) cases and were only detected among children under one (1) year old. It can caused gastroenteritis that may be complicated by hemorrhagic colitis or hemolytic–uremic syndrome. Authors reported that EHEC was predominantly present in the environment and reservoirs, but it did not play role in infantile diarrhoea (Khan *et al.*, 2002). However, our finding of EHEC in symptomatic infection, suggests that this pathotype should be monitored in future in infantile diarrhoea. Moreover, the study conducted by Kagambèga *et al.* (2013) show a high prevalence of EHEC in cattle feces, showing the source of distribution of the pathogen in developing countries where basics hygiene in water and foods is not required yet.

EIEC were detected in 2 (8, 7%) cases. The low frequencies of EIEC strains is also in agreement with other studies performed in different parts of the world (Nguyen *et al.*, 2005; Moyo *et al.*, 2007; Bonkoungou *et al.*, 2012; Hegde *et al.*, 2012). EIEC invades the colonic epithelium, allowing cell-to-cell spread of the bacteria. Invasion is mediated by the genes located in virulence plasmid pINV coding, e.g. Ipa proteins and their transcription regulator *invE.* Because of these factors,

EIEC is considered as the most important pathotype among DEC (Kaper et al., 2004). In this study, the prevalence of ETEC was 4, 35% (1 case). This is similar to the finding reported by Hegde *et al*. (2012), where the rate of ETEC was 3,5%. ETEC is recognized as a leading cause of diarrhoea in countries with inadequate sanitary conditions and among traveller's to those countries (Qadri *et al*., 2005). However, several authors have reported that ETEC and EIEC pathotypes are becoming more and rarer (Lozer *et al*, 2013; Ali *et al*, 2012), this could explained the low level of detection of these pathotypes in our study.

Of the 23 patients who carried the virulence genes, 17 (74%) were children under 5 years old. Other authors reported similar prevalence in children under five year of old (Al-Ghamdi *et al*., 2009; Zhou *et al*., 2013; Dembélé et al., 2015). This high prevalence of DEC in patients at a young age could be explained by the fact that this age group would be most vulnerable to diarrheal diseases due to the low immunity. The most affected population is children between 0 and 24 months old as observed in Sudan (Saeed *et al*., 2015) and in Burkina Faso (Dembélé *et al*., 2015). The rate of the diarrhoea was higher in male children, than in female patients with diarrhoea, as reported in many previous studies (Moyo *et al*., 2011; Sherchand *et al*., 2009). This could be explained by the fact that male children are more active in moving and touching many things than female children.

CONCLUSION

Diarrhoea is an important cause of mortality and morbidity in different areas of the world and among all age groups. The findings of this investigation provide updated information on pathotypes of DEC and their distribution by age and sex in Ouagadougou, Burkina Faso. It also indicated that the five major DEC pathotypes persist and circulate to variable rates in Ouagadougou, Burkina Faso. However, EAEC is the predominant DEC isolated from the examined patients with diarrhoea. Further studies are needed to investigate the epidemiology, pathogenicity and virulence properties of EAEC strains.

Ethical considerations

Permission to conduct this study was obtained from the hospital authorities of Burkina Faso and the study protocol was approved by the Ethical Committee(s) of Burkina Faso. Informed verbal consent was also obtained from patients before taking the stool samples.

Competing of interests

The authors declare that they have no competing interests.

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