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PREVALENCE OF *ESCHERICHIA COLI* VIRULENCE GENES IN PATIENTS WITH DIARRHOEA IN OUAGADOUGOU, BURKINA FASO

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ABSTRACT

Objective: **Diarrhoeagenic *E. coli* (DEC) strains are important causes of diarrhoea in the developing world and, to a lesser extent, inthe developed world. In this study, we investigated the prevalence of the virulence genes specific for five major pathogroups of diarrheagenic *Escherichia coli* (DEC) in primary cultures from diarrhoeagenic patients in Burkina Faso.*Methodology:* From September 2016 to Mars 2017, a total of 211 faecal samples from diarrhoeagenic patients from urban hospitals of Ouagadou, Burkina Faso have been analysed. A 16-plex PCR was used to detect simultaneously, the five major DEC pathotypes (enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), Shiga toxin-producing *E. coli* (STEC), enteroaggregative *E. coli* (EAEC) and enteroinvasive *E. coli* (EIEC)).**

Results: **At least one diarrhoeagenic *E. Coli* pathotype was detected in 31 samples (14.7%) in children and adults with diarrhoea. EAEC was the most common pathotype detected 9.5% (20/211), followed by EIEC2.4% (05/211) and STEC 0.5% (01/211). More than one DEC pathotype were detected in 2.4% (05/211) patients. EPEC and ETEC were not detected in single infection but in co-infection with others pathotypes.**

Conclusion: **DEC, especially enteroaggregative, may be important responsible of diarrhoeas in Burkina Faso from all ages patient.**

Key Words: Diarrhoeagenic *Escherichia coli*, 16-plex PCR, Burkina Faso, human diarrhoeas stool.

PREVALENCE DES GENES DE VIRULENCE D'*ESCHERICHIA COLI*ISOLEES DES SELLES DIARRHEIQUES CHEZ LES PATIENTS A OUAGADOUGOU, AU BURKINA FASO.

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RESUME

Objectifs: Les *E. coli* diarrhéiques (ECD) sont la cause des diarrhées chez les enfants comme chez les adultes dans les pays en développement. Dans cette étude nous avions pour objectif d'évaluer la présence des cinq principaux pathogroups ECD, leur association avec la diarrhée chez les enfants et les adultes au Burkina Faso.

Méthodologie: De Septembre 2016 à Mars 2017, un total de 211 échantillons de selles diarrhéiques a été recueilli chez des patients dans 2 centres de santé à Ouagadougou. La PCR 16-plex a été utilisée pour détecter la présence de *E. Coli* enteroagrégrégative (ECEA), *E. Coli* enteropathogène (ECEP), *E. Coli* enterotoxigénique (ECET), *E. Coli* entérohémorragique (ECEH) et*E. Coli* enteroinvasive (ECEI).

Résultats: Au moins un pathovarde *E. coli* diarrhéique a été détecté dans 31 échantillons (14,7%) chez les enfants et les adultes atteints de diarrhée. ECEA était le pathhotype le plus fréquent détecté à 9,5% (20/211), suivi de ECEIà 2,4% (05/211) et en fin de ECSTà 0,5% (01/211). ECEP et ECET n'ont pas été détectées en elles seules mais elles ont été détectées en co-infection avec d'autres pathotypes.

Conclusion:ECD, en particulier *E. coli*entero-aggregatif, pourrait être le plus redouté des agents responsables des diarrhées chez les personnes de tous âges au Burkina Faso.

Mots clés : ***Escherichia coli*** **Diarrhéique, PCR 16-plex, Burkina Faso, selles diarrhéiques.**

INTRODUCTION

Diarrhoea remains a leading cause of mortality and morbidity worldwide, particularly in developing countries [1]. The aetiological agents include a wide range of viruses, bacteria and parasites [2]. Among bacterial pathogens, diarrhoeagenic*Escherichia coli* (DEC) is an important agent of endemic and epidemic diarrhoea worldwide [3]. *Escherichia coli (E. coli)* is a heterogeneous group of typically non-pathogenic gram-negative bacteria, which are a natural partof the intestinal flora of animals and humans [4, 5]. However,the pathogenic strains are associated with several diseases,including diarrhoea, urinary tract infections and meningitis[6]. Diarrhoeagenic*E. coli* (DEC) strains are important causesof diarrhoea in the developing world and, to a lesser extent, inthe developed world [3].Diarrheal illness causes much mortality worldwide, particularly in children under the age of 5 [7]and particularly in countries in sub-Saharan Africa and South Asia, where children suffer many diarrhoea-related deaths. While there are many etiological agents responsible for diarrhoea, pathogenic *E. coli* is major contributor [8, 9].Diarrheal disease morbidity in children living in underserved countries is a leading cause of mortality andmorbidity in children living in underserved countries [9]. Close to 2 millions of children below 5 years of age are estimated to die every year because of diarrheal diseases[1].*E. coli* strains involved in intestinal infections in humans are classified into six (06) pathotypes:enteropathogenic*E. coli* (EPEC), *Shiga toxin-producingE. coli* (STEC) (e.g., *enterohemorrhagicE. coli* [EHEC]), *Shigella/enteroinvasiveE. coli* (EIEC), *enteroaggregativeE. coli* (EAEC), diffusely adherent *E. coli* (DAEC), and *enterotoxigenicE. coli* (ETEC). Globally, EPEC are most frequently causeof infantilediarrhoea.STEC is much more present from ruminant animals and in the environment. Different plants consumed by humans may be contaminated by of STEC, either by fertilization from contaminated animals, or when contaminated water is used for irrigation.EAEC were most responsible to traveller's diarrhoea. To date, only one report on DEC rotavirus detected from children with and without diarrhoea in urban and rural Burkina Faso has been published [2]. In this study, we investigated the prevalence of the virulence genes specific for five major pathogroups of DEC, theirs association with diarrhoea from both children and adults in Burkina Faso.

MATERIAL AND METHODS

Samples collection: From September 2016 to Mars 2017, 211diarrhoeas stools samples were collected in 2 health centers of Ouagadougou:Laboratoire National de Santé Pulique (LNSP) and Hopital du District de Bogodogo (HDB). These samples compound 194 to LNSP and 17to HDB. The samples were transported to the laboratory and kept at 4°C until the microbiological examination.All microbiological and molecular tests were carried out at LNSP Ouagadougou, Burkina Faso.

Cultivation samples: The stool samples were cultured onto sorbitol MacConkay agar and incubated at 37° C overnight. Bacterial mass on the sorbitol MacConkey agar plates was collected and stored for further analysis at -30°C in tubes with 1 mL brain-heart infusion broth containing 15% (volume/volume) glycerol.

16-plex PCR assay:The presence of STEC, EPEC, ETEC, EIEC and EAEC in human diarrhoeal stoolssamples was detected by 16-plex PCR for the genes *uidA, pic, bfp, invE, hlyA, elt, ent, escV, eaeA, ipaH, aggR, stx1, stx2, estIa, estIb*and *astA*. The primers and PCR conditions were as previously described [10]. 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 *stx1* and/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 a EPEC); 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*.

For DNA extraction a loop ful of bacterial growth was taken from the first streaking area of the plate. It was suspended into 250 µl of sterile water in an Eppendorf tube, boiled at 100 °C for 10 min, and centrifuged.

PCR was performed in a reaction of 20 µl containing 2.5µl 10 X PCR buffer (SolisBiodyne), 0.75µl dNTPs (10mM), 0.25 µl MgCl2 (50 mM), 0.2µl Taq DNA polymerase (5 U/µl), 0.5µl of an each

mixture of the 16 primer pairs at the concentrations listed in Table1, 12.8 µl of PCR-grade water and 2.5 µl of DNA sample was added to bring the final volume to 10µl. The cycling conditions used in the thermal cycler (Applied Biosystem, 2720 thermal cycler, Singapore) were 98 °C for 30 s, 35 cycles of 98 °C for 30 s, 63 °C for 60 s, 72 °C for 90 s with a final extension at 72°C for 10 min.

The amplified PCR products were separated by agarose gel (2% w/v) electrophoresis and visualized under UV light (Bioblock Scientific, Illkirch, CEDEX) after staining with ethidium bromide. Reference strains RHE 4283 (E 2348/69) for EPEC, FE94725 (Burkina Faso, beef) for ETEC, FE102301 (Burkina Faso, beef) for STEC, RHE 6647 (145-46-215, Statens Serum Institute [SSI], Copenhagen, Denmark) for EIEC, IH 56822 (patient isolate [11], for EAEC, and FE95562 (Burkina Faso, beef) for STEC-ETEC were included in each PCR run. All the 16-plex PCR positive results were confirmed by single PCRs.

Statistical analysis

Excel and the ANOVA tests or the chi-square test were used to determine the statistical significance of the data. A value of $p<0.05$ indicated statistical significance.

Ethical considerations

Permission to conduct this study was obtained from the hospital authorities of Burkina Faso and informed verbal consent was obtained from adults' patients and the parents/guardians of every child before taking the stool samples.

RESULTS

Clinical specimens

A total 211 diarrhoeas stools sample whose 194 to LNSP and 17 to HDB were collected during September 2016 to march 2017 from two big hospital centers in Ouagadougou, Burkina Faso. This collection concerned only children under five years to HDB and all ages to LNSP. One hundred and ten (110) stool samples (52.1%) were collected from males and 101 (47.9%) from female. Forty four percent (93/211) were children under five years, 15% (32/211) were young people to 6 years to 18 years, and40.8% (86/211) were adult patients over 19 years of age.

TABLE 1: OLIGONUCLEOTIDE PRIMERS USED FOR DETECTION OF THE VIRULENCE GENES

Pathovars	Genes	Sequences	bp	Concentration	reference
STEC, EPEC	*eae*-F	TCAATGCAGTTCCGTTATCAGTT	482	0.1	[12]
	eae-R	GTAAAGTCCGTTACCCCAACCTG		0.1	
	escV-F	ATTCTGGCTCTCTTCTTCTTTATGGCTG	544	0.4	[13]
	escV-R	CGTCCCCTTTTACAAACTTCATCGC		0.4	
	ent-F	TGGGCTAAAAGAAGACACACTG	629	0.4	[13]
	ent-R	CAAGCATCCTGATTATCTCACC		0.4	
Typical EPEC	*bfpB*-F	GACACCTCATTGCTGAAGTCG	910	0.1	[13]
	bfpB-R	CCAGAACACCTCCGTTATGC		0.1	
STEC	*hlyEHEC*-F	TTCTGGGAAACAGTGACGCACATA	688	0.1	[10]
	hlyEHEC-R	TCACCGATCTTCTCATCCCAATG		0.1	
	stx1A-F	CGATGTTACGGTTTGTTACTGTGACAGC	244	0.2	[13]
	stx1A-R	AATGCCACGCTTCCCAGAATTG		0.2	
	stx2A-F	GTTTTGACCATCTTCGTCTGATTATTGAG	324	0.4	[13]
	stx2A-R	AGCGTAAGGCTTCTGCTGTGAC		0.4	
EIEC	*ipaH*-F	GAAAACCCTCCTGGTCCATCAGG	437	0.1	[10]
	ipaH-R	GCCGGTCAGCCACCCTCTGAGAGTAC		0.1	[14]
	invE-F	CGATAGATGGCGAGAAATTATATCCCG	766	0.2	[13]
	invE-R	CGATCAAGAATCCCTAACAGAAGAATCAC		0.2	
EAEC	*aggR*-F	ACGCAGAGTTGCCTGATAAAG	400	0.2	[13]
	aggR-R	AATACAGAATCGTCAGCATCAGC		0.2	
	pic-F	AGCCGTTTCCGCAGAAGCC	1111	0.2	[13]
	pic-R	AAATGTCAGTGAACCGACGATTGG		0.2	
	astA-F	TGCCATCAACACAGTATATCCG	102	0.4	[13]
	astA-R	ACGGCTTTGTAGTCCTTCCAT		0.4	
ETEC	*LT*-F	GAACAGGAGGTTTCTGCGTTAGGTG	655	0.1	[13]
	LT-R	CTTTCAATGGCTTTTTTTTGGGAGTC		0.1	
	STIa-F	CCTCTTTTAGYCAGACARCTGAATCASTTG	157	0.4	[13]
	STIa-R	CAGGCAGGATTACAACAAAGTTCACAG		0.4	
	STI-F	TGTCTTTTTCACCTTTCGCTC	171	0.2	[13]
	STI-R	CGGTACAAGCAGGATTACAACAC		0.2	
E. coli	*uidA*-F	ATGCCAGTCCAGCGTTTTTGC	1487	0.2	[13]
	uidA-R	AAAGTGTGGGTCAATAATCAGGAAGTG		0.2	

STEC= Shiga toxin-producing *E. coli*; EPEC= enteropathogenic*E. coli*; EIEC= enteroinvasive*E. coli*; EAEC= enteroaggregative*E. coli*; ETEC= enterotoxigenic*E. coli*.

Detection of DEC Multi-plex PCR (16-plex PCR) was used to detect virulence genes carried by diarrheagenic*E. coli* and to classify the strains as STEC, EPEC, ETEC, EIEC, or EAEC. At least one diarrhoeagenic*E. coli*pathotype was detected in 31 samples (14.7%) in patientswhose 14% (27/194) at LNSP and 23.5% (04/17) at HDB.Globally DEC pathogroups were detected to 14.7% (31/211) including 11.8% (13/110) from male and 17.8% (18/101) from female.EAEC was the most common pathotype detected 9.5% (20/211), followed by EIEC2.4% (05/211) and STEC 0.5% (01/211) (**Table 2 and figure 1**). EPEC and ETEC were not detected in single infection but in co-infection with others pathotypes. One EPEC isolates was typical as EPEC isolates was only positive for *bfpA*and negative for *eae*. This EPEC was co-infection with EAEC.No atypical EPEC were detectedfor all stool samples analysed. Five DEC co-infections were detected to all stools samples analysed (**Table 2**). Considering the different ages groups, DEC pathogroups were detected in 18.9% (07/37) from children under five years, whose 8.1% (03/37) EAEC, 8.1% (03/37) EAEC and one co-infection to EAEC + ETEC (**figure 2**).One or more DEC pathogroups were detected in (6.2%) 12/194 patient more than five years all from LNSP. Therewere significant differences in DEC prevalence betweenchildren under five years and adults ($p<0.0001$).

TABLE 2: DETECTION OF DIARRHOEAGENIC *ESCHERICHIA COLI* PATHOVARS FROM CHILDREN AND ADULTS WITH DIARRHOEA IN OUAGADOUGOU, BURKINA FASO

Pathovars/aeras/sex	LNSP n=194 (%)	HDB n=17 (%)	M n=110 (%)	F n=101 (%)	Total n=211 (%)
EAEC	20 (10.3)	00	08 (7.3)	12 (11.9)	20 (9.5)
EIEC	02 (01.3)	03 (17.6)	03 (2.3)	02 (1.9)	05 (2.4)
STEC	01 (0.5)	00	00	01 (1.0)	01 (0.5)
EAEC + EIEC	01 (0.5)	00	01 (0.9)	00	01 (0.5)
EAEC + ETEC	00	01 (5.9)	00	01 (1.0)	01 (0.5)
EEAC + STEC	02 (01.0)	00	01 (1.0)	01 (0.9)	02 (0.9)
EAEC + t-EPEC	01 (0.5)	00	00	01 (1.0)	01 (0.5)
Total	27 (14.0)	04 (23.5)	13 (11.8)	18 (17.8)	31 (14.7)

STEC= Shiga toxin-producing*E. coli*; EPEC= enteropathogenic*E. coli*; EIEC= enteroinvasive*E. coli*; t-EPEC= typical-enteropathogenic*E. coli*; ETEC= enterotoxigenic*E. coli*, LNSP= Laboratoire National de Santé Publique, HDB= Hôpital de District de Bogodogo, % percentage, n= number, M= males, F= females

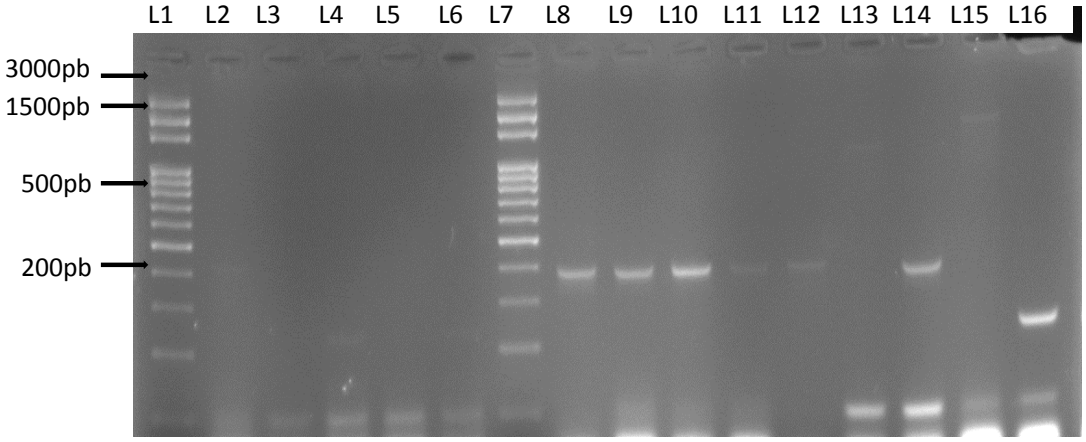

FIGURE 1:EXAMPLE OF THE 16-PLEX PCR RESULTS TO DEC ISOLATED FROM HUMAN DIARRHEAS STOOLS SAMPLES IN LNSP AND HDB.L1=marker, L2= RHE6647 (EIEC), L3=IHE56822 (EAEC), L4=FE102301 (STEC), L5=FE94725 (ETEC), L6=FE95562 (STEC-ETEC), L7=marker, L8=CpI-73, L9=Cp349, L10=Cp354, L11=Cp393, L12=Cp419, L13=Cp435, L14=CpI-99, L15=CpI-02, L16 =Cp63

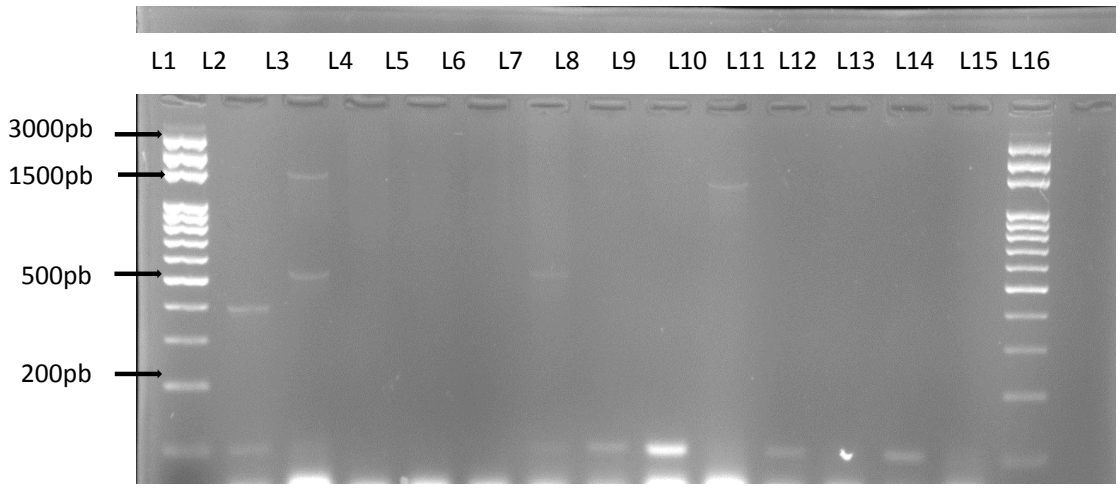

FIGURE 2: EXAMPLE OF THE 16-PLEX PCR RESULTS TO DEC ISOLATED FROM HUMAN DIARRHEAS STOOLS SAMPLES (FOLLOWING) L1=marker, L2= HDB535, L3= HDB536, L4=HDB540, L5=HDB541, L6=Cp88, L7=marker, L8=Cp91, L9=Cp95, L10=Cp96, L11=Cp111, L12=Cp113, L13=Cp120, L14=Cp121, L15=marker,L16 =Water (negative control).

DISCUSSION

Diarrhoea is an important cause of mortality and morbidity in different areas of the world (developed and developing countries) and among all age groups [15].The epidemiology of enteric pathogens that cause diarrhoeasuggests that most infections are acquired from food, water,and hand contact and many diarrheal diseases can be prevented by simple rules of personal hygiene and safer foodpreparation[15, 16].

In this study, from all stool samples analysed; DEC were detected in 14.7 %of cases, of which 8.5 % were from children under five yearsand 6.2 % from adults. These results were similar with others study's results in Africa and Brasilia [17, 18, 19], however in 2012 Bonkoungou*et al.* [2] have detected 45% of DEC from diarrheal stool samples from children under five years in urban and rural hospital centers in Burkina Faso.Our studyshowed that different pathovars DEC were more found to children under five years than adults patients.This could be explained by the fact that in children under five years old the immune system is much more fragile whereas *E. coli* leaves the flora in adults. Diarrheal diseases caused by DEC are most often linked to the hygienic conditions of the environment in which we live. Indeed, these results indicate a change in the respect of good hygiene practices in the population. Moreover, it can be said that the objectives set by the hygiene services on raising public awareness of good hygiene practices are half achieved. Nonetheless, sensitization must continuate to improve hygiene in water supplies and kitchens, and better sanitation is necessary to reduce the incidence of gastro-enteric infections, including DEC infections in Burkina Faso.Moreover, with the epidemic of Ebola in West Africa, populations have incorporated hand washing systems or systematic disinfection of the hands. That was not the case in 2012.

EAEC is an emerging pathogen associated with diarrhoea. It has been identified in travellers, children in the developing world and human immunodeficiency virusinfected patients with diarrhoea[20, 21]. In the present study, EAEC strains were the most frequently isolated pathotype of *E. Coli*confirming earlier reports of EAEC's role as a human pathogen [21, 22].This is a strong indication that this pathotype occur widely in the hospital center in Burkina Faso.Studies conducted in many countries havedemonstrated the importance of EAEC in hospital centers. This might be due to the poor hygienicpractices and very low standards of living indeveloping countries, and it implies that improvementsin sanitary conditions and water quality can beeffective control measures.

STEC are mostly associated with outbreaks and two important diseases, including haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) [23]. Cattle are perceived as their primary reservoir and are the major source of food contamination [24, 25]. In this study low frequency of STEC and EIEC (one only) was detected, but co-infections was found with EAEC. We can hypothesize that our strains (diarrheal stool samples) analysedwere not related tohaemorrhagic colitis and haemolytic uremic syndrome. Similar results were found in sub-Saharan Africa and others countries [2, 26, 27, 28].

ETEC causes a significant number of cases of childhooddiarrhoea and gastroenteritis among travellers. ETEC were also the most common agent of traveller'sdiarrhoea with food and water implicated as the modes of transmission [3]. In our study, we did not found ETEC but co-infections was found with EAEC. However Bonkoungou*et al*. in Burkina Faso showed the presence of ETEC in the children with diarrhoea. ETEC were detected on important proportion in others study conducted in sub-Saharan Africa countries [3, 5, 9, 19].

EPEC is also a very important pathogen in children with diarrhoea. EPEC infection is primarily a disease of infants younger than 2 years of age. Numerous case–control studies in many countries have found that EPEC is more frequently isolated from children with diarrhoea than from healthy controls [2, 3].However, in our study, the detection rate of EPEC strains from adults with diarrhoea was low (only one typical EPEC) and it co-infection with EAEC. In this study no EPEC was detected from children under five years.

The subjectsemployed in this study may be infected by otherpathogens other than diarrhoeagenic*E. coli*since there are different pathogens that cancausediarrhoea mostly in children, including Rotavirus,*Salmonella* spp., *Shigella*spp., *Campylobacterjejuni*, *Entamoeba histolytica,* and *Giardia lamblia*[5].

ConclusionThe multiplex PCR assay used in our study amplifying 16 virulence genes in a single reaction, is a practical and fast tool for DEC identification in routine clinical diagnostic and epidemiological studies.This study showed the diversity of *E. coli* virulence gene expression in two big hospital centers to Burkina Faso. It suggests that DEC is an importantdiarrhegenic agent and should be considered in routinestudies and surveillance for childhood acute diarrhealdisease.Hand washing with soap and good hygienic practices in the families and public places could contribute to decrease DEC infection.

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