Diarrhoeagenic *Escherichia coli* detected by 16-plex PCR in children with and without diarrhoea in Burkina Faso

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Abstract

The importance of diarrhoeagenic *Escherichia coli* (DEC) in Africa is poorly understood, and is unknown in Burkina Faso. This study investigated the occurrence of five major DEC pathogroups in primary cultures of stool samples from 658 Burkinabe children under 5 years old using 16-plex PCR for virulence-associated genes. At least one DEC pathogroup was detected in 45% of 471 children with diarrhoea and in 29% of 187 children without diarrhoea ($p < 0.001$). More than one DEC pathogroup was detected in 11% of children with and 1% of children without diarrhoea ($p < 0.001$). Enteroaggregative *E. coli* was the most common pathogroup in both children with diarrhoea (26%) and children without diarrhoea (21%). Enteropathogenic *E. coli* and enterotoxigenic *E. coli* were detected significantly more often in children with diarrhoea (16% and 13%) than in children without diarrhoea (5% and 4%; $p < 0.001$ for both pathogroups). Shiga toxin-producing *E. coli* and enteroinvasive *E. coli* were detected only in children with diarrhoea (2% and 1%, respectively). Diarrhoeagenic *E. coli*, especially enteropathogenic and enterotoxigenic, may be important, unrecognized causes of childhood diarrhoea in Burkina Faso.

Keywords: 16-plex PCR, Burkina Faso, diarrhoeagenic *Escherichia coli*
Original Submission: 27 May 2011; **Revised Submission:** 5 August 2011; **Accepted:** 7 September 2011
Editor: M. Drancourt
Article published online: 15 September 2011
Clin Microbiol Infect 2012; **18:** 901–906
10.1111/j.1469-0691.2011.03675.x

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Introduction

Diarrhoeal disease is a major public health problem throughout the world, with over two million deaths occurring each year, affecting mostly children under 5 years of age in developing countries [1,2]. The aetiological agents include a wide range of viruses, bacteria and parasites. Among bacterial pathogens, diarrhoeagenic *Escherichia coli* (DEC) are important agents of endemic and epidemic diarrhoea worldwide [3].

The DEC can be divided into several pathogroups based on their distinct virulence determinants: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), Shiga toxin-producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC) and enteroaggregative *E. coli* (EAEC) being the most important [4]. EPEC is further divided into two subtypes, typical and atypical EPEC, depending on the presence or absence of bundle-forming pili. Strains of atypical EPEC occur most frequently in developed countries, whereas typical EPEC are the leading cause of infantile diarrhoea in developing countries [5]. ETEC is considered to be a major cause of traveller's diarrhoea in adults from industrialized countries and the leading cause of infant diarrhoea in developing countries. ETEC causes diarrhoea by producing heat-labile and heat-stable enterotoxins [3,6]. Strains of STEC cause acute inflammation in the intestine and possibly

bloody diarrhoea with rare but severe secondary complications such as haemolytic uraemic syndrome. The main virulence factors of STEC are Shiga toxins, which induce cytotoxic effects on the microvascular endothial cells [7].

Strains of EIEC invade the colonic epithelium and cause bloody diarrhoea and symptoms that resemble those caused by *Shigella* spp. Invasion is mediated by the genes located in virulence plasmid pINV encoding, for example, Ipa proteins and their transcription regulator *invE* [3,8]. EAEC harbours the mechanism for an aggregative-adherence pattern mediated by aggregative adhesive fimbriae. It is increasingly recognized as a diarrhoeal pathogen in developing countries [9].

The identification of DEC pathogroups requires their differentiation from non-pathogenic members of the normal human intestinal *E. coli* flora and conventional microbiological testing cannot be used for conclusive identification. To date, routine detection and identification of DEC are usually based on a combination of biochemical tests, serotyping and molecular methods.

The importance of DEC in Africa is poorly understood and unevenly studied because of the lack of facilities needed to characterize them. No up-to-date information is available on their role in childhood diarrhoea in Burkina Faso.

Therefore, we investigated the occurrence of the five major DEC pathogroups in children aged below 5 years with and without diarrhoea in urban and rural Burkina Faso, using a 16-plex PCR method for the detection of their virulence genes.

Materials and Methods

Study design and sampling

The stool sample collection was conducted between July 2009 and January 2010 in three regions in Burkina Faso: Ouagadougou (125 children with and 50 children without diarrhoea), which is the capital city located in the centre of Burkina Faso, Gourcy (153 children with and 77 children without diarrhoea) and Boromo (193 children with and 60 children without diarrhoea), which are rural areas located in northern and western parts of the country (Fig. 1). The latter regions are tropical savannah areas and the main sources of income in this rural setting are subsistence farming, animal husbandry and small scale trade.

Samples were collected from 471 children under the age of 5 years who had diarrhoea (defined as three or more loose, liquid, or watery stools or at least one bloody loose stool in 24 h [10]) and/or were admitted to the pediatric service of Centre Médical du Secteur 30 of Ouagadougou or the Medical District Hospital of Gourcy or Boromo. The control subjects, randomly selected during the same period, were 187 children with no history of diarrhoea for at least 21 days coming to the same health centre for reasons other than diarrhoea. Demographic information (age and location) and clinical symptoms (fever, vomiting and dehydration) were recorded for each child using a questionnaire. Samples were taken by trained healthcare personnel using a swab transport system (M40 transystem Amies Agar Gel Without Charcoal; Copan Italia Spa, Brescia, Italy) and transported to the National Public Health Laboratory in Ouagadougou within 24 h of their collection.

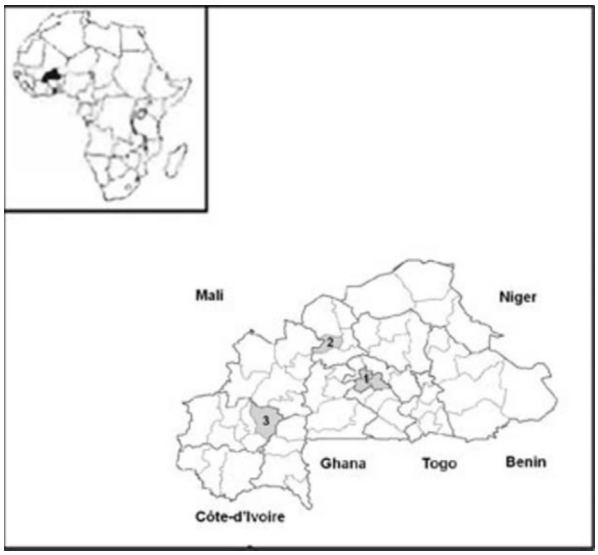

FIG. 1. Map of Burkina Faso showing the locations where the sampling was performed. (1) Ouagadougou, the capital city located in the central part, (2) Boromo and (3) Gourcy in rural settings located in western and northern part of Burkina Faso.

Cultivation of samples

The stool samples were cultured onto sorbitol MacConkey 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 for DEC

The fragments of 16 genes were amplified with specific primers in a single PCR for detection of the virulence genes of DEC (Table 1) as previously described [11].The criteria for determination of a DEC were as follows: for EPEC, the presence of *eaeA* and *escV* and possible additional genes *ent* and *bfpB*, the absence of *bfpB* indicated atypical EPEC; for ETEC, the presence of *elt* and/or *estIa* or *estIb*; for STEC, the presence of stx_1 and/or stx_2 and possible additional genes *eaeA*, *escV*, *ent* and EHEC-*hly*; for EIEC, the presence of *invE* and

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

Pathotypes	Target gene	Primer sequence (5′–3′)	Product size (bp)	Concentration (μM)	References
STEC, EPEC	*eaeA*	eae-F: TCAATGCAGTTCCGTTATCAGTT	482	0.1	[28]
		eae-R: GTAAAGTCCGTTACCCCAACCTG		0.1	
	escV	MP3-escV-F: ATTCTGGCTCTCTTCTTCTTTATGGCTG	544	0.4	[30]
		MP3-escV-R: CGTCCCCTTTTACAAACTTCATCGC		0.4	
	ent	ent-F: TGGGCTAAAAGAAGACACACTG	629	0.4	[30]
		ent-R: CAAGCATCCTGATTATCTCACC		0.4	
Typical EPEC	*bfpB*	MP3-bfpB-F: GACACCTCATTGCTGAAGTCG	910	0.1	[30]
		MP3-bfpB-R:CCAGAACACCTCCGTTATGC		0.1	
STEC	EHEC-*hly*	hlyEHEC-F: TTCTGGGAAACAGTGACGCACATA	688	0.1	[11]
		hlyEHEC-R: TCACCGATCTTCTCATCCCAATG		0.1	
	stx1	MP4-stx1A-F:CGATGTTACGGTTTGTTACTGTGACAGC	244	0.2	[30]
		MP4-stx1A-R: AATGCCACGCTTCCCAGAATTG		0.2	
	stx2	MP3-stx2A-F:GTTTTGACCATCTTCGTCTGATTATTGAG	324	0.4	[30]
		MP3-stx2A- R: AGCGTAAGGCTTCTGCTGTGAC		0.4	
EIEC	*ipaH*	ipaH-F: GAAAACCCTCCTGGTCCATCAGG	437	0.1	[11]
		ipaH-R: GCCGGTCAGCCACCCTCTGAGAGTAC		0.1	[29]
	invE	MP2-invE-F: CGATAGATGGCGAGAAATTATATCCCG	766	0.2	[30]
		MP2-invE-R: CGATCAAGAATCCCTAACAGAAGAATCAC		0.2	
EAEC	*aggR*	MP2-aggR-F: ACGCAGAGTTGCCTGATAAAG	400	0.2	[30]
		MP2-aggR-R: AATACAGAATCGTCAGCATCAGC		0.2	
	pic	MP2-pic-F: AGCCGTTTCCGCAGAAGCC	1,111	0.2	[30]
		MP2-pic-R: AAATGTCAGTGAACCGACGATTGG		0.2	
	astA	MP2-astA-F: TGCCATCAACACAGTATATCCG	102	0.4	[30]
		MP2-astA-R: ACGGCTTTGTAGTCCTTCCAT		0.4	
ETEC	*elt*	MP2-LT-F: GAACAGGAGGTTTCTGCGTTAGGTG	655	0.1	[30]
		MP2-LT-R: CTTTCAATGGCTTTTTTTTGGGAGTC		0.1	
	estIa	MP4-STIa-F:CCTCTTTTAGYCAGACARCTGAATCASTTG	157	0.4	[30]
		MP4-STIa-R: CAGGCAGGATTACAACAAAGTTCACAG		0.4	
	estIb	MP2-STI-F: TGTCTTTTTCACCTTTCGCTC	171	0.2	[30]
		MP2-STI-R: CGGTACAAGCAGGATTACAACAC		0.2	
E. coli	*uidA*	MP2-uidA-F: ATGCCAGTCCAGCGTTTTTGC	1,487	0.2	[30]
		MP2-uidA-R: AAAGTGTGGGTCAATAATCAGGAAGTG		0.2	

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

ipaH, for EAEC, the presence of *pic* and/or *aggR*. The gene *uidA* was used as a general marker for *E. coli*. The gene *astA* was not specific for a certain pathogroup so it was not used in the final analysis. Any unclear PCR results were verified using a single primer pair PCR assay.

For the PCR, a loopful of bacteria from the thickly growing area of a sorbitol MacConkey agar plate was transferred to an Eppendorf tube with 250 μL water. The mixture was boiled for 10 min and centrifuged for 1 min at 11337 ***g*** and the supernatant was analysed in the PCR reactions. One microlitre of the supernatant was added to 9 μL reaction mixture containing 0.16 U *Taq* DNA polymerase (Finnzymes, Espoo, Finland), 2 μL of 5 $\times$ GC buffer, 0.24 μL 25 mM $MgCl_2$, 0.4 μL of a mixture of the four DNA deoxynucleotide triphosphates (5 mM each) and PCR primers at the concentrations listed in Table 1. Thermocycling conditions were as follows: 98°C for 30 s, 35 cycles of 98°C for 10 s, 62.5°C for 60 s and 72°C for 90 s, with a final extension at 72°C for 10 min. Amplified DNA fragments were separated by agarose gel electrophoresis (1.5% weight/volume), stained with ethidium bromide and visualized under UV light. Reference strains EPEC RH 4283 (E 2348/69, [12]), ETEC RH 4266 (ATCC 35401), STEC RH 4270 (ATCC 43895), EIEC RH 6647 (145–46–215; Statens Serum Institute, Copenhagen, Denmark), EAEC IH 56822 (patient isolate, [13]) and the *E. coli*-negative control RH E6715 (ATCC25922) were included in each PCR run.

Statistical analysis

The chi-square test or Fisher's exact test of OpenEpi version 2.3.1 was 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 the parents/guardians of every child before taking the stool samples. The study protocol was approved by the Ethical Committees of Burkina Faso and the Hospital District of Helsinki and Uusimaa, Finland.

Results

At least one DEC pathogroup was detected in 214 of 471 children with diarrhoea (45%) and in 54 of 187 children without diarrhoea (29%) ($p < 0.001$) (Table 2). More than one DEC pathogroup was detected in 52 (11%) children with

TABLE 2. Detection of diarrhoeagenic *Escherichia coli* pathogroups from 471 children with diarrhoea and 187 control children (without diarrhoea) from urban and rural Burkina Faso

	No. (%) of DEC detected in stool samples			
	Children with diarrhoea (n = 471)	Children without diarrhoea (n = 187)	Odds ratio (95% CI)*	p value (χ^2)
Children with any DEC	214 (45.4)	54 (28.9)	2.1 (1.4–2.9)	<0.001
Children with no DEC	257 (54.6)	133 (71.1)	2.1 (1.4–2.9)	<0.001
DEC pathogroup (all)	271 (57.5)	56 (29.9)	3.2 (2.2–4.6)	<0.001
EPEC	77 (16.3)	10 (5.3)	3.5 (1.7–6.8)	<0.001
tEPEC	19 (4.0)	2 (1.1)	3.9 (0.9–16.9)	NS
aEPEC	58 (12.3)	8 (4.2)	3.1 (1.5–6.7)	0.002
ETEC	61 (12.9)	7 (3.7)	3.8 (1.7–8.5)	<0.001
ST	33 (7.0)	4 (2.1)	3.4 (1.2–9.9)	0.015
LT	20 (4.2)	3 (1.6)	2.7 (0.8–9.3)	NS
ST+LT	8 (1.7)	0	NA	NS
STEC	8 (1.7)	0	NA	NS
EIEC	3 (0.6)	0	NA	NS
EAEC	122 (25.9)	39 (20.6)	1.3 (0.9–2.9)	NS
DEC co-infections	52 (11.0)	2 (1.1)	11.5 (2.8–47.6)	<0.001
EAEC+EPEC	19 (4.0)	1 (0.5)	7.8 (1.0–58.8)	0.02
EAEC+ETEC	18 (3.8)	1 (0.5)	7.4 (1.0–55.8)	0.02
EAEC+STEC	4 (0.8)	0	NA	NS
EPEC+ETEC	4 (0.8)	0	NA	NS
EIEC+ETEC	2 (0.4)	0	NA	NS
EAEC+EPEC+ETEC	5 (1.0)	0	NA	NS

DEC, diarrhoeagenic *E. coli*; EPEC, enteropathogenic *E. coli*; tEPEC, typical EPEC; aEPEC, atypical EPEC; ETEC, enterotoxigenic *E. coli*; ST, heat-stable enterotoxin-producing strain; LT, heat-labile enterotoxin-producing strain; STEC, Shiga toxin-producing *Escherichia coli* ; EIEC, enteroinvasive *E. coli*; EAEC, enteroaggregative *E. coli*.
*CL, confidence interval; NA, not applicable; NS, not significant.

diarrhoea and in two (1%) children without diarrhoea (p <0.001). As a result of the simultaneous occurrence of two or three DEC pathogroups in some stool samples, a total of 271 DECs were detected in the 214 children with diarrhoea and 56 DECs were found in the 54 children without diarrhoea (Table 2).

The most common pathogroup in both children with 122/471 (26%) and children without 39/187 (21%) diarrhoea was EAEC (p >0.05). Among the children with EAEC, the *aggR* gene was the most commonly detected gene, in 83% of the children with and in 77% of children without diarrhoea.

EPEC and ETEC were detected significantly more often in children with diarrhoea, 77/471 (16%) and 61/471 (13%), respectively, than in children without diarrhoea 10/187 (5%) and 7/187 (4%) (p <0.001 for both findings). Of the EPEC findings, 58/77 (75%) in children with diarrhoea and 8/10 (80%) in children without diarrhoea were classified as atypical EPEC because of the lack of the *bfpB* gene. Among the 61 ETEC findings in children with diarrhoea, samples carrying only *estIa* and/or *estIb* genes were more prevalent, 33/61 (54%), than those carrying the *elt* gene, 20/61 (33%), or both *elt* and *estIa or estIb*, 8/61 (13%).

A low number of STEC and EIEC were detected in children with diarrhoea, eight (2%) and three (1%), but they were absent in samples collected from children without diarrhoea (Table 2).

One or more DEC pathogroups were detected in 92/153 (60%) children with diarrhoea from Gourcy, 83/193 (43%) from Boromo and 39/125 (31%) from Ouagadougou (Table 3). The difference was statistically significant between the two rural locations Gourcy and Boromo (p <0.05) and between the rural locations and the capital city (Gourcy and Ouagadougou, p <0.001; Boromo and Ouagadougou, p <0.05).

Considering the different age groups (Table 3), DEC pathogroups were detected in all of them, but most cases (81%) occurred among children <2 years old. EAEC was mainly detected among children under the age of 1 year, EPEC in children aged younger than 2 years and ETEC in

TABLE 3. Clinical and epidemiological features among the 214 children with diarrhoea and one or more DEC pathogroups detected (%)

	DEC pathogroups						
Epidemiological and clinical characteristics	EPEC n = 46	ETEC n = 32	STEC n = 4	EIEC n = 2	EAEC n = 78	Co-infection n = 52	Total n = 214
Origin (Region)							
Ouagadougou	11 (24)	5 (16)	0 (0)	1 (50)	14 (18)	8 (15)	39 (18)
Boromo	23 (50)	6 (19)	2 (50)	0 (50)	35 (45)	17 (33)	83 (39)
Gourcy	12 (26)	21 (65)	2 (50)	1 (50)	29 (37)	27 (52)	92 (43)
Age in months							
0–12	20 (43)	8 (25)	0 (0)	0 (0)	46 (59)	24 (46)	98 (46)
13–24	19 (41)	14 (44)	1 (25)	1 (50)	22 (28)	18 (35)	75 (35)
25–59	7 (15)	10 (31)	3 (75)	1 (50)	10 (13)	10 (19)	41 (19)
Diarrhoea type							
Watery	43 (93)	28 (87)	3 (75)	1 (50)	72 (92)	46 (88)	193 (90)
Bloody	3 (7)	4 (13)	1 (25)	1 (50)	6 (8)	6 (12)	21 (10)
Symptoms							
Fever ≥38°C	36 (78)	25 (78)	3 (75)	1 (50)	50 (64)	40 (77)	155 (72)
Vomiting	17 (37)	9 (28)	2 (50)	0 (0)	40 (51)	13 (25)	81 (38)
Dehydration	11 (24)	3 (9)	1 (25)	1 (50)	15 (19)	8 (15)	39 (18)

DEC, diarrhoeagenic *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; STEC, Shiga toxin-producing *Escherichia coli* ; EIEC, enteroinvasive *E. coli*; EAEC, enteroaggregative *E. coli*.

children older than 1 year. Among children with diarrhoea who were positive for DEC, 193/214 (90%) had watery diarrhoea and 21/214 (10%) had bloody diarrhoea. Bloody diarrhoea was more common in the STEC and EIEC infections than in the infections with the other DEC pathogroups (Table 3). However, only a few STEC and EIEC cases were detected. Fever was the symptom most commonly reported in association with DEC diarrhoea (72%), followed by vomiting (38%) and dehydration (18%).

Discussion

In this study, the first of its kind in Burkina Faso, we investigated the occurrence of five major DEC pathogroups by 16-plex PCR in 658 stool samples from 471 children with diarrhoea and 187 children without diarrhoea. The prevalence of DEC (one or more pathogroups present) was significantly higher in children with diarrhoea (45%) than in those without (29%). Similar results have also been reported in southeastern and western Africa, e.g. in Mozambique and Nigeria [14,15]. In our study, EAEC was the most commonly detected DEC pathogroup in both groups of children, suggesting that EAEC is not associated with diarrhoea, as also suggested by other recent studies [13,16]. However, some researchers have found EAEC to be associated with diarrhoea [17,18]. The conflicting results might result from the heterogeneity of the EAEC strains, differences in the infection load, variability in susceptibility of the individuals or the use of different characterization methods. In support of our results are reports from Nigeria and Ghana, close neighbours of Burkina Faso, which indicate that EAEC is endemic among children in local communities [19,20], and might not be a primary cause of diarrhoea.

The most frequently detected DEC associated with diarrhoea was EPEC. The atypical EPEC was the most commonly detected EPEC in children both with and without diarrhoea, which indicates that atypical EPEC is more prevalent than typical EPEC in Burkina Faso. In accordance with our findings, numerous case–control studies from various countries have found that EPEC is associated with diarrhoea [3,4]. Atypical EPECs are commonly isolated from animals, in contrast to typical EPEC [3]. In Burkina Faso, the close association between humans and the domestic and farm animals could play an important role in human infection.

In the present study, the prevalence of ETEC was 13% in the children with diarrhoea and it was highly significantly associated with diarrhoea. Other researchers [21,22] have reported rates similar to ours. In addition, a recent review on DEC in sub-Saharan Africa [23] stated that ETEC is strongly associated with infantile diarrhoea in African countries and also the most common cause of acute diarrhoea. In Burkina Faso, as has been shown in other studies elsewhere, heat-stable enterotoxin-producing ETEC are more strongly associated with childhood diarrhoea than are heat-labile enterotoxin-producing strains, even though the latter may be more common overall [19,24,25].

The low frequencies of STEC and EIEC were expected [14,17,21,26]; however, infection with STEC in children can potentially lead to a severe disease and complications, such as haemolytic uraemic syndrome, so it warrants special attention.

In this study, mixed co-infections of different DEC pathogroups were common and were found also in children without diarrhoea, making it difficult to define the exact aetiology of diarrhoea in ill children. Especially the role of EAEC in the mixed infections can be questioned based on the common occurrence of EAEC in children without diarrhoea.

Our results show that DEC were more common in rural settings (43% in Boromo and 60% in Gourcy) than in Ouagadougou (31%). Living conditions in the rural settings differ from those of the capital city, where food hygiene and quality of water sources for human consumption are at a higher level. In rural areas people and their animals live in the same space. These conditions may explain the higher frequency of DEC pathogroups detected in the rural settings. When the association of the DEC pathogroups with age and clinical features were examined, DEC were observed in children of all ages under 5 with an increased occurrence in children aged 6–12 months and those 1–2 years of age, as observed also in Tunisia [27]. Bloody diarrhoea was less common than watery diarrhoea probably because the number of patients with EIEC and STEC, which are commonly associated with bloody diarrhoea, was low. Also the other clinical symptoms were, in general, similar regardless of the detected DEC pathogroup, as has been reported elsewhere [26].

The 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. The diagnostics afforded by DEC give useful information about the aetiology of diarrhoeal diseases and may help to implement mechanisms for disease control and prevention. Our data suggest that diarrhoeagenic *E. coli*, especially EPEC and ETEC, are an important, though still unrecognized, cause of childhood diarrhoea in Burkina Faso.

Authors' Contributions

IB and RD were responsible for collection of the stool samples and clinical information as well as data analysis. IB, TL

and OM conducted the 16-plex PCR assays. IB, TL, OM, IS, AT, AS, NB and KH participated in designing the study and writing the manuscript. KH and NB designed the whole research collaboration.

Acknowledgements

We thank the parents and guardians of the studied children and the Boromo, Gourcy and Ouagadougou sector 30 Health District Authorities for their cooperation. These results were partially presented at the 9th International Meeting on Microbial Epidemiological Markers (IMMEM-9), September 2010 in Wernigerode, Germany.

Transparency Declaration

This study was funded by the Academy of Finland grant 122600 for the collaboration between the Finnish National Institute for Health and Welfare (THL) and the CRSBAN/UFR-SVT (University of Ouagadougou), Burkina Faso. All the authors declare that they have no conflict of interest.

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