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Research Article

Molecular Diagnosis of *Shigella*, *Salmonella* and *Campylobacter* by Multiplex Real-Time PCR in Stool Culture Samples in Ouagadougou (Burkina Faso)

Salfo Sawadogo, Msc, Engineer[1,2], Birama Diarra, PharmD, Msc, PhD Student[1], Cyrille Bisseye, PhD, Assistant Professor[1,3], Tegwindé Rebeca Compaore, PhD, assistant researcher[1,4], Florencia W. Djigma, PhD, Assistant Professor[1,4], Djénéba Ouermi, PhD, assistant Professor[1,3], Aboubakar S. Ouattara, PhD, Full Professor[5], and Jacques Simporé, PhD, Full Professor[1,4]

[1]Pietro Annigoni Biomolecular Research Centre, CERBA/LABIOGENE, University of Ouaga I Pr Joseph KI-ZERBO, 01 BP 364 Ouagadougou 01, Burkina Faso
[2]El Fateh SUKA Clinic, 04 BP 8297 Ouagadougou 04, Burkina Faso
[3]Laboratory of Molecular and cellular Biology (LABMC), University of Sciences and Techniques of Masuku (USTM), BP 943 Franceville, Gabon
[4]Saint Camille Medical Centre, 01 BP 364 Ouagadougou 01, Burkina Faso
[5]CRSBAN, University of Ouaga I Pr Joseph KI-ZERBO, 03 BP 7131 Ouagadougou 03, Burkina Faso

Corresponding Author: Cyrille Bisseye; email: cbisseye@gmail.com

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Abstract

Background: Bacteriological diagnosis of *Campylobacter* spp, *Salmonella* spp and *Shigella* spp could be necessary in case of infectious gastroenteritis syndrome.
The objective of this study was to diagnose concomitantly the three enteropathogenic bacteria by multiplex Real-Time PCR in stool culture samples in Ouagadougou (Burkina Faso). **Materials and Methods**: The study was conducted from February 5th to March 9th, 2013. Two hundred stool samples were received during the study period. The bacteria were identified by bacterial culture following by multiplex Real-Time PCR. **Results:** *Shigella* spp and *Campylobacter* spp were sought by culture in all 200 samples. Enteropathogenic *E. coli* was sought only in 37 samples from all children under 2 years old. The bacterial culture was positive in 12 stool samples. *Shigella* spp and *Salmonella* spp. were isolated respectively in 5 (2.5%) and 3 samples (1.5%). Enteropathogenic *E. coli* was isolated in 10.8% (4/37) of the samples tested. The multiplex real-time PCR identified bacteria in 20 patients, including 17 cases of *Shigella* spp., 1 case of *Salmonella* spp. and 2 cases of *Campylobacter* spp. **Conclusions**: This study has highlighted the low frequency of 3 sought bacterial genera in stool samples. It has also demonstrated a significant difference between the culture and the multiplex Real-Time PCR method in the diagnosis of *Shigella*.

Keywords: Stool culture; Real-time PCR; *Shigella* spp.; *Salmonella*. spp. ; *Campylobacter* spp. Burkina Faso

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1. Authors' Contribution

Study concept and design: Dr Cyrille Bisseye and Prof Jacques Simpore

Collection of data: Salfo Sawadogo and Dr Birama Diarra

Analysis and interpretation of data; Salfo Sawadogo, Dr Birama Diarra, Dr Cyrille Bisseye and Dr Tegwindé Rebeca Compaoré

Drafting the article and revision: Salfo Sawadogo, Dr Cyrille Bisseye, Dr Birama Diarra, Dr Djeneba Ouermi, Dr Florencia W. Djigma, Dr Tegwindé Rebeca Compaoré,

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Funding: Prof Jacques Simpore

From: Dr. Cyrille Bisseye

Pietro Annigoni Biomolecular Research Centre

CERBA/LABIOGENE

University of Ouaga I Pr Joseph KI-ZERBO,

01 BP 364 Ouagadougou 01, Burkina Faso

Burkina Faso, West Africa

Fax: +226 50 30 23 28

Email: cbisseye@gmail.com

2. Introduction

Each year, there are about two billion cases of diarrheal diseases worldwide causing the death of more than 2 million people, including 1.9 million children under the age of 5 mainly in developing countries [1]. In Burkina Faso, diarrhea is responsible for 12% of deaths in children under 5 years old [2]. Among the pathogens involved in these infections, enteric bacteria such as *Campylobacter*, *Salmonella* and *Shigella* are important because of the frequency and severity of the symptoms they could cause. Thus, *Campylobacter* is currently considered the leading cause of intestinal bacterial infections in humans worldwide with an increasing incidence in developed countries [3–5]. Although, *Campylobacter* infection is usually mild, the increase in cases of campylobacteriosis, the existence of rare but serious complications such as abortions, peritonitis, meningitis, Guillain-Barre syndrome and the alarming increase of *Campylobacter* antibiotic resistance explain the renewed interest in this bacterial genus [3]. Regarding *Salmonella*, the World Health Organization (WHO) estimates about 17 million cases of typhoid fever in the world with more than 500,000 deaths and 1.3 billion annual cases of gastroenteritis due to non-typhoid *Salmonella* with 3 million deaths annually [6].

As for *Shigella*, it is the leading cause of malnutrition in developing countries where shigellosis remains endemic [7]. The cases of antibiotics' multi-resistant bacteria at high levels are also reported for *Salmonella* and *Shigella* [8–11]. The direct diagnosis of these three bacteria can be done by stool culture. *Campylobacter* diagnosis is generally not performed in bacteriology laboratories in Burkina Faso. In this country, no study has yet reported the molecular diagnosis of *Shigella*, *Salmonella* and *Campylobacter* in human stool samples. The objective of this study was to diagnose by multiplex real-time PCR, *Shigella*, *Salmonella* and *Campylobacter*in routine stool culture samples in Ouagadougou.

3. Materials and Methods

3.1. Sample Collection

A total of 200 stool culture samples were collected, including 101 samples from El Fateh Suka clinic and 99 at Saint Camille Medical Centre in Ouagadougou between February 5th and March 9th, 2013. For each stool sample, a portion of the sample was transferred into a sterile tube and stored at -80°C for molecular diagnostics. The rest was used for stool culture process within 2 h of receipt.

Patients or parents and/or guardians of under-age children answered a questionnaire including their socio-demographic and clinical characteristics.

3.2. Stool Culture

Salmonella, *Shigella* and enteropathogenic *E. coli* (EPEC) were sought on the basis of existing repositories [12]. For all samples, the Hektoen agar Petri dish and *Salmonella* enrichment broth (Selenite broth) were inoculated and incubated in an oven at 37°C. After 5 to 6 hours, selenite broth was sub-cultured on a second Hektoen agar plate. Both Hektoen boxes were then incubated in an oven at 37°C for 18 to 24 hours.The suspected colonies were biochemically identified by API 20E (Biomérieux®, Marcy-Etoile, France) and also sub-cultured on Kligler-Hajna medium. *Salmonella* and *Shigella* colonies identified on Kligler-Hajna medium were agglutinated with *Salmonella* and *Shigella* antisera (Bio-Rad®, Marnes-la-Coquette, France) for confirmation and species or serotype identification. The detection of EPEC (Enteropathogenic *E. coli*) was carried out on the samples of the children under 2 years old. Its identification was performed on EMB agar. Typical colonies were agglutinated with an Enteropathogenic *E. coli* antisera (Bio-Rad®, Marnes-la-Coquette, France).

3.3. Sample Preparation, Extraction and Amplification of Bacterial Genomes

After thawing the samples, 1 mL (for liquid stool) or 1 gram (for solid faeces) was used for the extraction of bacterial DNA using the DNA-Sorb-B extraction kit (Sacace Biotechnologies®, Como, Italy) according to the manufacturer's instructions.

The bacterial genomes were amplified by multiplex real-time PCR using the kit "Shigella/Salmonella/Campylobacter Real-TM" (Sacace Biotechnologies®, Como, Italy). The amplification reaction was performed in a SaCycler-96 Real-Time PCR (Sacace Biotechnologies®, Como, Italy) according to the manufacturer's recommendations. The target genes were respectively *IpaH*, *TtrB*, and *23SrRNA* for *Shigella*, *Salmonella* and *Campylobacter*.

3.4. Statistical Analysis

The results were analyzed using SPSS 17.0 Software and Epi Info version 3.5.1. The chi-square test was used for comparisons and the difference was considered significant for p-value < 0.05.

4. Results

4.1. Epidemiological and Clinical Characteristics of Patients

The stools were predominantly from female patients (60.5%), outpatients (94.5%) and subjects over 15 years (57.5%). The major clinical features of patients were respectively, fever (44.5%), diarrhea (21%) and vomiting (13%). The presence of blood and leucocytes in stool was observed in 2.5% and 5% of patients, respectively. Some of the patients (14.5%) had started antibiotic therapy before sample collection. The epidemiological and clinical characteristics of the patients are shown in Table 1.

4.2. Identification of Bacteria from Stool Specimens by Culture and Multiplex Real Time PCR

The number and percentage of germs diagnosed by the two diagnostic methods are shown in Table 2. Enteropathogenic *Escherichia coli* was sought only in samples of children aged less than 2 years was isolated 4 times by culture (4/37, 10.8%)

The prevalence of the three bacteria detected by multiplex real-time PCR was higher than that detected by stool culture; but the observed difference was not statistically

significant (10% vs 6%, p = 0.140). Bacteria prevalence detected by multiplex real time PCR and by stool culture were respectively, 8.5% vs 2.5% for *Shigella* and 0.5% vs 1.5% for *Salmonella*. *Shigella* prevalence detected by PCR was significantly higher compared to that detected by culture (p = 0.006). The distribution of *Shigella* species identified in the 5 patients was as follows: 3 cases of *S. flexneri*, 1 case of *S. boydii* and 1 case of *S. dysenteriae*.

The 3 cases of *Salmonella* identified were represented by three different serotypes: *Salmonella* Typhi, *Salmonella* Paratyphi *B* and *Salmonella* sp. of the 3 positive samples by culture, two were negative by PCR. *Campylobacter* spp. was identified by PCR only in 1% of patients (Table 2). The distribution of positive samples for the three bacteria by PCR based on epidemiological and clinical features of the patients is summarized in Table 3.

The distribution of *Shigella* spp. among patients was not associated with age, neither with sex or fever. The prevalence of *Shigella* was higher among patients with diarrheal compared to non-diarrheal patients (23.8% vs 4.4%; p < 0.001).

5. Discussion

This study aimed the concomitant search for *Shigella, Salmonella*, and *Campylobacter*by multiplex real-time PCR in stool samples from patients in Ouagadougou. To our knowledge, this is the first study on the molecular diagnosis of these three bacteria in Burkina Faso. However, the diagnosis of these pathogens by traditional methods has been reported in previous studies in children with diarrhea [13–17]. The prevalence of *Shigella*, *Salmonella* and *Campylobacter* by multiplex real-time PCR was 8.5%; 0.5% and 1%, respectively. This reduced prevalence of enteropathogenic bacteria could be explained the weak frequency of diarrhoeal patients among the stool specimens tested. Diarrhea is the major cause of stool specimens' culture [12, 18]. This suggests that in Burkina Faso, stool culture is done as well as parasites stool examination in the systematic search for certain gastrointestinal disorders in primary care. Diarrheas were more common in infants than adults (68.6% vs 12.2%). Similar results have been reported in Burkina Faso [13, 14] and Brazil [20]. Despite this high rate of diarrhea, bacteria were isolated only in 28.6% of infants. This could be explained by the fact that, viruses are the leading cause of diarrhea in children [12, 20]. In addition, the period of the study coincided with the cold dry season where the frequency of virus is highest among children with diarrhea in Burkina Faso [16, 17]. Bloody stool samples having a positive leukocyte microscopy examination, were all positive by culture and by PCR, which is in agreement with the entero-invasive nature of these bacteria [12].

Characteristics		Number	Percentage (%)
Age Group (years)			
	<2	37	18.5
	2-15	48	24.0
	>15	115	57.5
Sex			
	Male	79	39.5
	Female	121	60.5
Type of Patient			
	In-Patient	11	5.5
	Out-Patient	189	94.5
Diarrhea			
	Yes	42	21.0
	No	158	79.0
Fever			
	Yes	89	44.5
	No	111	55.5
Vomitting			
	Yes	26	13.0
	No	174	87.0
On Antibiotherapy *			
	Yes	29	14.5
	No	155	85.5
Stoolconsistency			
	Liquid	12	6.0
	Semi-liquid	23	11.5
	Hard	165	82.5
Bloody Stools			
	Yes	5	2.5
	No	195	97.5
leucocytesin stools			
	Yes	10	5.0
	No	190	95.0

Table 1: Patients' epidemio-clinic characteristics. *: Total number of answers = 184.

	Real-Time PCR n = 200		Bacterial Culture n = 200	
Germs	N	%	N	%
Salmonella	1	0.5	3	1.5
Shigella	17	8.5	5	2.5
Campylobacter	2	1.0	ND	ND
Total	20	10		

Table 2: Bacterial culture and PCR results summary. ND = not done.

	Number of bacteria detected by PCR			
Characteristics		**Shigella**	**Salmonella**	**Campylobacter**
Age group (years)				
	N	N (%)	N (%)	N (%)
<2	37	5 (13.5)	0	0
2-15	48	3 (6.3)	0	2 (4.2)
>15	115	9 (7.8)	1 (0.9)	0
Sex				
Male	79	8 (10.1)	0	2 (2.5)
Female	121	9 (7.4)	1 (0.8)	0
In-patient				
No	11	2 (18.2)	0	0
Yes	189	15 (7.9)	1 (0.5)	2 (1.1)
On Anti-biotherapy				
No	155	13 (8.4)	1 (0.6)	2 (1.3)
Yes	29	4 (13.8)	0	0
Diarrhea				
No	158	7 (4.4)	1 (0.6)	1 (0.6)
Yes	42	10 (23.8)	0	1 (2.4)
Fever				
No	111	8 (7.2)	1 (0.9)	1 (0.9)
Yes	89	9 (10.1)	0	1 (1.1)
Stool Consistency				
Liquid	12	5 (41.7)	0	0
Semi-liquid	23	5 (21.7)	0	2 (8.7)
Hard	165	7 (4.2)	1 (0.6)	0
Bloody stool				
No	196	14 (7.1)	0	0
Yes	4	3 (75.0)	0	0
Leucocytes in stools				
No	190	8 (4.2)	1 (0.5)	1 (0.5)
Yes	10	9 (90.0)	0	1 (10.0)

Table 3: Distribution of positive cases by PCR according to the patients' epidemio-clinic criterea.

Shigella was the most identified bacteria by stool culture as well as multiplex real-time PCR. Culture has identified 5 cases of shigellosis. *Shigella flexneri* was the most frequently isolated (3 of 5 cases), which is consistent with previous studies which have shown that it is the most common species in developing countries [16, 17, 21, 22].

The frequency of *Shigella* spp. was higher by PCR than stool culture. This difference should be interpreted with caution. Indeed, the PCR target gene *IpaH* which allowed the detection of *Shigella* is also present in the entero-invasive *E. coli* (EIEC).Thus, the highest frequency of *Shigella* by PCR could be due to the presence of EIEC in stool of

patients. To our knowledge, there is no molecular technique to differentiate *Shigella* from EIEC [23].

Thus, 5 samples were found positive for *Shigella* in culture and by the PCR technique. For 12 samples negative in culture but positive by PCR, the presence of the EIEC could not be excluded; although a recent study in Burkina Faso showed a very low frequency of EIEC (0.3%) compared to enteroaggregative *E. coli* (12%) and enteropathogenic *E. coli* (8%) [17].

The prevalence of *Shigella* spp. showing a discrepancy between the results of PCR and culture have been reported in Vietnam [24]. The present report and previous studies in Vietnam [24], India [25] and Thailand [26] suggest that stool culture has a lower sensitivity than PCR in the diagnosis of *Shigella* spp.

Regarding *Salmonella*, of the 3 positive samples in stool culture, two were PCR negative. The absence of *Salmonella* in negative PCR samples suggests either a lower specificity of culture (biochemical and antigenic similarity to other bacteria) or a lower sensitivity of PCR in the diagnosis of *Salmonella* compared to stool culture. A low sensitivity of PCR compared to culture in the detection of *Salmonella* was reported by Cunningham et al, [27]. However, because of the reduced size of our sample, additional data are needed to confirm one or the other of the two hypotheses.

The frequency of *Campylobacter* (1%) found in this study is similar to those reported in Burkina Faso [17] and France [28]. A study combining enzyme immunoassay methods, molecular and culture had reported a 9.5% rate in hospitalized patients with gastrointestinal problems [29]. The detection of *Campylobacter* by stool culture has been recommended by some studies [3, 28] and repositories [12]. It may be necessary for the diagnosis of certain enteritis and diarrhea.

6. Conclusion

This study has highlighted the low prevalence of the three bacteria diagnosed in routine stool culture samples. It also demonstrated a significant difference between multiplex real-time PCR and stool culture in the diagnosis of *Shigella*.

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