Evaluation of a Real-Time Polymerase Chain Reaction for the Laboratory Diagnosis of *Giardia intestinalis* in Stool Samples from Schoolchildren from the Centre-Ouest and Plateau Central Regions of Burkina Faso

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Abstract

Objective: Giardiasis, a zoonotic, diarrhoeal disease with worldwide occurrence, is routinely diagnosed by microscopic examination of stool samples. However, implementation of this method relies on skilled personal, it is time consuming and relatively low in sensitivity. A superior diagnostic approach to detect the causative agent *Giardia intestinalis* would, hence, be highly desirable. The current study aimed to assess real-time polymerase chain reaction (PCR) for the detection of *G. intestinalis* as an alternative to microscopy.

Material and methods: Stool samples from healthy schoolchildren, aged 8-14 years, from eight schools of the Centre-Ouest and Plateau Central regions in Burkina Faso were collected within a cross-sectional study in February 2015. Microscopic examination was performed on two faecal samples collected over two consecutive days from 441 schoolchildren. Each faecal specimen was examined using Kato-Katz and formol-ether methods of concentration in addition to direct examination. Real-time PCR was used to detect *G. intestinalis* in all microscopy-positive and a random sample of microscopy-negative samples.

Results: Microscopic examination revealed 94 microscopy-positive samples, and an overall *G. intestinalis* prevalence of 27.2%. Using the microscopic examination as the 'gold' standard, the overall sensitivity of real-time PCR was demonstrated to be 76.6% ranging from 58.3% to 94.1% and the specificity was 96.2% ranging from 96.2% to 100% across schools.

Conclusion: Real-time PCR appears to be a solid detection method for *G. intestinalis* in the current setting. However, it needs to be further optimized to become a more sensitive tool for *G. intestinalis* diagnosis in low-income settings.

Keywords: *Giardia intestinalis*; Molecular diagnosis; Stool; Burkina Faso

Introduction

Giardiasis is a zoonotic disease that affects at least 280 million people worldwide each year [1]. It is one of the main causes of non-viral diarrhoea in industrialized countries [2] and it is associated with additional digestive disorders in children and adults, including abdominal pain and nausea. Giardiasis can develop into a chronic condition, and disease can be aggravated in immunocompromised hosts. Infection with the causative intestinal protozoa *Giardia intestinalis* is commonly associated with unsafe drinking water, poor hygiene and sanitation, poverty and warm climate [3,4]. Correspondingly, the prevalence of *G. intestinalis* is estimated at 2-7% in developed countries and up to 20-30% in low- and middle-income countries (LMICs) [5]. Owing to their common faecal-oral mode of transmission, *G. intestinalis* is often found in coinfection with other intestinal protozoa [6].

Studies among school-aged children across Africa are scarce; however, those available report *G. intestinalis* prevalences of 16% in urban and 24% in rural areas of Morocco [7] and 11.7% in a rural setting in southern Ethiopia [8]. From Côte d'Ivoire, a 17.3% and 13.9% prevalence was demonstrated in the Man area [9] and the region of Agboville, respectively [6]. *G. intestinalis* prevalence in school-children in Burkina Faso is currently described by several smaller studies reporting on local numbers, while the national prevalence remains unknown. One such study, conducted in 2014, has shown

G. intestinalis prevalences of 13.3%, 12.5% and 9.8% among children aged 6-15 years from three different schools of the Central region [10]. Another setting revealed a prevalence of 43.7% in patients aged 5 months to 72 years suffering from gastroenteritis visiting the Saint Camille Hospital of Ouagadougou [11]. A retrospective study of parasitological aetiology assessments of gastroenteritis patients from the same hospital showed an infection rate of 24.8% [12].

Metronidazole that has been on the market since 1959 [13] is the main drug used to treat giardiasis. However, growing resistance of *G. intestinalis* to this drug has been reported [13] and no new drug is available to efficiently treat the disease. To minimize the extent of resistance development, it is strongly recommended to treat only diagnostically confirmed cases while avoiding blind or presumptive treatment [14]. There is currently no 'gold' standard for the diagnosis of giardiasis [15]. However, the most common approach is the microscopic examination of trophozoïtes or cysts of *G. intestinalis* in stools samples [16]. Challenges of this microscopy-based diagnosis include low sensitivity (around 60% for single testing due to intermittent faecal excretion of the parasite) and a high dependence on the skills and experience of the technician [16]. Previous efforts on identifying alternative means of *G. intestinalis* diagnosis include the detection of parasite antibodies by direct immunofluorescence and immune-enzymatic methods (e.g. ELISA). However, as with microscopy, these techniques require experienced technicians along with specialized equipment [17]. A biochemical assay was also tested for the potential diagnosis of giardiasis, based on the detection of volatile organic compounds that derive from the protozoan metabolism, in faecal samples. This technique is not feasible in routine laboratory, because it requires sophisticated equipment [18]. Strain characterization using molecular biology techniques such as real-time polymerase chain reaction (PCR) has proven to be highly sensitive and specific for *G. intestinalis* [19]. Yet, the use of PCR in parasitology is limited in African laboratories compared with its application in bacteriology or virology [19]. Many studies have focused on the use of PCR to test samples assessed as negative by microscopy, but very few were dedicated to the verification of positive cases of *G. intestinalis* in its cystic form. There is a particular need for the verification of microscopy-positive samples owing to the difficulty of differentiating *G. intestinalis* cysts from non-pathogenic intestinal protozoa and Morel spores when the technician is not highly trained [20].

The objective of this study was to assess real-time PCR to detect *G. intestinalis* in microscopy-positive stools samples from healthy schoolchildren in the Centre-Ouest and Plateau Central regions of Burkina Faso.

Methods

Study area and population

This study was conducted in February 2015 in the frame of the 'Vegetable go to School: improving nutrition through agricultural diversification (VgtS)' project in Burkina Faso [21]. Four hundred and forty-one schoolchildren aged 8-14 years were randomly selected from four schools in the Centre-Ouest region (Douré A, Goundi B, Ipendo A and Tio A) and four schools of the Plateau Central region (Loumbila A, Linoghin A, Tangzougou and Wavoussé).

These regions have a Sudano-Sahelian type of climate, and consist predominantly of agricultural areas and small farms. Questionnaires were administered to schoolchildren, their teachers and parents in order to determine the socio-demographic characteristics of the schoolchildren, and the socioeconomic conditions in the corresponding schools and households. The nutritional status of the schoolchildren was assessed by measuring anthropometric parameters on the first day of stool collection.

Collection of microscopy-positive and microscopy-negative stool samples

Two fresh morning stool samples were collected from each child on two consecutive days, into a clean and dry container with an identification label. The stool samples were subsequently transported in coolers or thermostats containing ice boxes to the IRSS's microbiology laboratory for parasitological analysis. The transport was conducted in the shortest possible time (in any case less than two hours) to avoid a temperature rise which might lyse the protozoan trophozoïtes. First, macroscopic examination of the stool was conducted to document the consistency and the potential presence of blood, mucus and macroscopic parasites. Second, the stool samples were analysed by direct microscopic examination of fresh sample and of sample concentrated by the formalin-ether sedimentation method of Ritchie [22], to detect intestinal protozoa and helminths (roundworm eggs are commonly destroyed by this technique). A total of 147 stool samples (corresponding to all 94 microscopy-positive and 53 microscopy-negative samples for *G. intestinalis*) were stored at -80°C prior real-time PCR analysis. As in previous molecular studies, we have been working on cysts that are more resistant than trophozoites [23].

Quality control of parasitological analysis was performed on 10% of the samples and conducted by a well-trained technician from the Teaching University Hospital Pediatric Charles de Gaulle, (Ouagadougou, Burkina Faso), who re-analysed the stool samples using microscopic-based examination. Quality control pertained to a total of 43 stools samples, including 9 positive and 34 negative stool samples for *G. intestinalis*, selected at random.

DNA extraction of *Giardia intestinalis*

DNA extraction from stool samples was conducted using the DNA-Sorb-B Laboratory kit of Sacace Biotechnologies® (Como, Italy). Samples were thawed at room temperature and pre-treated as follows: a suspension of each sample was prepared by introducing 1.0 ml of saline and 0.1 g of the stool sample into a polypropylene tube. The suspension was vortexed and then centrifuged at 10 000 g for 5 min. A volume of 100 µl of the pellet was subsequently transferred into an Eppendorf tube containing 800 µl of PBS buffer 10% and the supernatant removed after centrifugation. The pellet was suspended with 300 µl of PBS buffer 10%. The resulting mixture was used for the DNA extraction, which was performed according to the manufacturer's protocol (Sacace Biotechnologies®; Como, Italy). The extracted DNA was stored at -20°C prior to use.

Real-time PCR analysis

Real-time PCR was performed using thermal cycler 7500 Fast Real Time PCR System (Applied Biosystems/Life Technologie; California, USA). The total volume of the reaction mix for the amplification was 25 µl consisting of 12 µl of Taq Man Universal Master Mix, 3 µl of the probe (0.25 µM), 1 µl of each sense and anti-sense primer (50 µM) (Applied Biosystem, California, USA), 3 µl of water (DNAse-RNase free) and 5 µl of extracted DNA. The gene of the small subunit

ribosomal RNA (SSU rRNA) was amplified using a (5'-GAC GGA GGC ATT CAA CGG TT-3') forward primer and a (5'-TTG CCA TCC GCG GTG G-3') reverse primer. The amplicons were detected using the sequence FAM-CCC GCG GCG CTC GTC GCT AG-BHQ as probe. The DNA amplification programme was set up as previously describes by Mejia et al. [24], as follows: after preheating at 60°C for 1 min, a cycle was performed at 95°C for 10 min followed by 45 cycles at 95°C during 15 s, 60°C for 1 min and a final extension at 60°C for 1 min.

Statistical analysis

Data were entered into an Excel 2013 spreadsheet (Microsoft Corp, Redmond, USA) and analysed in SPSS version 20 (SPSS Inc.; Chicago, USA). Pearson's χ^2 test was used to study the association between *G. intestinalis* (microscopy) as the dependent variable and the PCR results and the socio-demographic and socioeconomic outcomes as independent parameters. To explore the presence of *G. intestinalis* (as assessed by real-time PCR) and associations with age, sex, years of schooling, nutritional status of schoolchildren, educational level of the household head, presence of a latrine in the residence and availability of drinking water, a binary logistic regression was performed. The odds ratio (OR) was estimated with a 95% confidence interval (CI) and P-values were calculated. The results were considered significant for P<0.05. Considering microscopy as 'gold' standard, the sensitivity and specificity of real-time PCR were assessed according the TDR Diagnostics Evaluation Expert Panel [25].

Ethical considerations

The study was conducted as part of the VgtS study [21], which received the approval of the 'Ethikkommission Nordwest- und Zentralschweiz' in Switzerland (EKNZ, reference no. 2014-161), the ethics committee for health research of Burkina Faso (reference no. 2015-02-026) and of the institutional ethics committee of CERBA/LABIOGENE, University of Ouaga I Joseph Ki Zerbo. The study is registered with the clinical trial registry ISRCTN (identifier: ISRCTN17968589).

Written informed consent of each care-givers was obtained, while children assented orally before starting the investigation. Study participation was voluntary and children could withdraw anytime without further obligation. All infected schoolchildren received free treatment according to national guidelines.

Results

Characteristics of the study population

The 147 schoolchildren enrolled for the real-time PCR study had an average age of 11 years (range 8 to 15 years), and 57.1% of them were male. Among the 94 schoolchildren with microscopy-positive results, 59.6% were male, 37.2% were malnourished, 40.4% had a latrine in their residence and 51.1% were in grade 4. Three of the children with confirmed giardiasis (3.2%) had diarrhoea in the last two weeks preceding the survey. Among the 53 schoolchildren with microscopy-negative results, 52.8% were male, 24.5% were malnourished, 62.3% had a latrine in their residence and 75.5% were in grade 4.

The nutritional status of the 147 children was evaluated using the body mass index (BMI) for age as defined by the World Health

Organization (WHO) in 2007 [26]. According to the corresponding WHO score, 32.7% (48/147) of the children were, per definition, underweight or malnourished, represented by 6/19 (31.6%), 4/13 (30.8%), 6/14 (42.9%) and 11/24 (45.8%) schoolchildren from the schools in Doure A, Goundi B, Ipendo A and A Tio in the Centre-Ouest region and 7/19 (36.8%), 4/19 (21.1%), 5/21 (23.8%) and 5/18 (27.8%) schoolchildren from the schools in Linoghin A, Loumbila A, Tangzougou and Wavoussé A in the Plateau Central region. No statistically significant association was found between malnutrition and school. The binary logistic regression showed that schoolchildren without access to a latrine in their residence had a higher prevalence of *G. intestinalis* than those with access (OR 1.7; 95% CI 0.8–3.8). This trend was, however, not significant.

Detection of *G. intestinalis* by direct microscopic examination of stool samples

According to the results of the microscopy-based diagnosis of stool samples from 441 schoolchildren, 120 children were found to harbour *G. intestinalis* (i.e. cystic forms or trophozoïtes), which represents an overall prevalence of 27.2%. Among them, 94 samples showed cystic forms of *G. intestinalis,* while 26 samples contained vegetative forms.

The prevalence of *G. intestinalis* was 33.9%, 27.8%, 23.5% and 30.2% in the schools of Doure A, Goundi B, Ipendo A and Tio A in the Centre-Ouest region and 20.0%, 30.9%, 19.6% and 23.2% in the schools of Linoghin A, Loumbila A, Tangzougou and Wavoussé in the Plateau Central region.

As previously reported, despite a varying intensity, *G. intestinalis* infection showed no statistically significant difference between schools (P=0.2) or between regions (P=0.1) [27]. Co- and multiple infections were significantly more frequent in boys (86%) than girls (77.1%) (P=0.038).

The rate of concordance between our results and those of the teaching hospital was very high, ie 95.3% (41/43). Indeed, of the 43 samples of which 9 positive and 34 negative to *G. intestinalis* that were subjected to this external quality control, one was negative and another one positive, respectively.

G. intestinalis and *Entamoeba histolytica/E. dispar* showed the strongest association (P=0.025) (Table 1), followed by *G. intestinalis* and *Escherichia coli* (34%). No significant association was found between the presence of multiple infections and underweight in schoolchildren (P=0.585).

The educational atteinment of the head of a household seemed to influence the infection prevalence among schoolchildren. A large proportion of the infected children (81.4%) was associated with households where the head had not received any formal schooling. A proportion of 12.4% of infected children belonged to parents that had received primary education and 6.2% were associated with parents that had completed secondary school. However, no statistically significant difference was found. A significant association was demonstrated for *G. intestinalis* presence and availability of a latrine at the household (P=0.002) which was the case in less than half of all households (39/97). Most of the households assessed (87/97) had access to drinking water and no difference was observed in the infection prevalence of schoolchildren with and without such access.

	Entamoeba histolytica/E. dispar		Escherichia coli		Trichomonas intestinalis		Hymenolepis nana	
	Present N (%)	Absent N (%)	Present N (%)	Absent N (%)	Present N (%)	Absent N (%)	Present N (%)	Absent N (%)
Giardia intestinalis (N=94)	60 (63.8)	34 (36.2)	32 (34.0)	62 (66.0)	26 (27.7)	68 (72.3)	7 (7.4)	87 (92.6)
P-value	0.025*		0.217		0.287		0.624	

**Table 1:** Coexistence of *Giardia intestinalis* and other frequent parasites; *significant.

Real-Time PCR						
Centre-Ouest Region						
				Pos	Neg	Total
Douré A	Microscopy	Pos		16	1	17
		Neg		0	2	2
		Total		16	3	19
Goundi B	Microscopy	Pos		7	1	8
		Neg		0	5	5
		Total		7	6	13
Ipendo A	Microscopy	Pos		9	1	10
		Neg		0	4	4
		Total		9	5	14
Tio A	Microscopy	Pos		9	4	13
		Neg		1	10	11
		Total		10	14	24
Total Centre Ouest Region	Microscopy	Pos		41	7	48
		Neg		1	21	22
		Total		42	28	70
Plateau Central Region						
Tangzougou A	Microscopy	Pos		6	1	7
		Neg		1	13	14
		Total		7	14	21
Wavousse	Microscopy	Pos		8	4	12
		Neg		0	6	6
		Total		8	10	18
Linonghin A	Microscopy	Pos		7	5	12
		Neg		0	7	7
		Total		7	12	19
Loumbila A	Microscopy	Pos		10	5	15

			Neg	0	4	4
			Total	10	9	19
Total Plateau Central Region	Microscopy		Pos	31	15	46
			Neg	1	30	31
			Total	32	45	77
Total Centre-Ouest and Plateau Central Regions						
Total Centre-Ouest and Plateau Central Regions	Microscopy		Pos	72	22	94
			Neg	2	51	53
			Total	74	73	147

Table 2: Sensitivity and specificity results of the real-time PCR method to detect *G. intestinalis* (overall sensitivity: 76.6; overall specificity: 96.2).

G. intestinalis detection by real-time PCR

The results of the DNA amplification of *G. intestinalis* cysts by real-time PCR are given in the form of fluorescence intensity curves expressed as a function of the number of cycles (Figure 1).
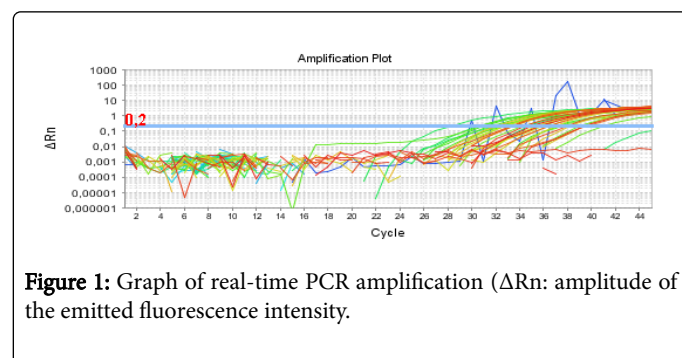
Figure 1: Graph of real-time PCR amplification (ΔRn: amplitude of the emitted fluorescence intensity.

Real-time PCR confirmed the presence of *G. intestinalis* DNA in 76.6% (72/94) of the microscopy-positive cases. The sensitivity of real-time PCR ranged from 69.2% to 94.1% in the Centre-Ouest region and 58.3% to 85.7% in the Plateau Central region (Table 2). Of the 53 microscopy-negative samples for *G. intestinalis*, 51 were confirmed as negative using real-time PCR. The overall specificity of the real-time PCR was 96.2% ranging from 95.4% in the Centre-Ouest region to 96.7% in the Plateau Central region (Table 2). No statistically significant difference was observed between a PCR positive status and

schools of the two regions. However, a significant difference (P=0.032) was observed for *G. intestinalis* prevalence according to region, with Centre-Ouest having a higher rate. Furthermore, no significant association was observed between *G. intestinalis* positive samples, as assessed by real-time PCR, and age, sex, nutritional status, years of schooling, the level of parental education, and availability of a latrine at home or availability of safe drinking water at home.

Discussion

In LMICs, the estimation of the prevalence of parasitic diseases is often complicated by the absence of reliable data owing to a lack of effective diagnostic tools. Despite being considered a major cause of parasite-related diarrhoea worldwide [28] and being included in the WHO 'Neglected Diseases Initiative' in 2004 [3], national prevalence estimates of *G. intestinalis* are still not available in many African countries.

The aim of this study was to evaluate the performance of real-time PCR in detecting *G. intestinalis* in microscopy-positive stool samples in Burkina Faso. We found an overall prevalence of *G. intestinalis* among school-children of 27.2%, which is in line with previous estimates of 20-30% in LMICs [5]. The children infected with *G. intestinalis* showed no apparent clinical signs of the disease which might be explained by a high proportion of cysts compared to trophozoïtes.

Given that microscopy is deemed a suitable diagnostic method for *G. intestinalis* infection, the sensitivity found in previous studies for PCR-based detection of *G. intestinalis* differs wildly and ranges from 13-100%. In the current study, we have found a specificity of PCR ranging from 91-100% across the eight different schools. High sensitivity of a PCR-based approach has been shown previously by Schuurman et al. who detected the presence of a single cyst by real-time PCR [29]. Within a study conducted in the Netherlands, 100% of 20 microscopy-positive stools were confirmed positive by multiplex PCR [30]. These authors had reported 10/34 real-time PCR positive on microscopy-negative samples.

Using real-time PCR, Verweij et al. detected 102 positive samples among 104 microscopy-positive samples that contained visible *G. lamblia* cysts, resulting in a sensitivity of 98.1% [19]. In Bangladesh Ng et al. reported 85% detection rate of *G. intestinalis* using multiplex real-time PCR from patients with diarrhoea [31]. Also, among subjects from Timor and Cambodia, Llewellyn et al. observed a detection rate of 65.7% (46/70) using multiplex real-time PCR [32]. In Morocco, from 84 samples only 11 were PCR positive (13.1%) using semi-nested PCR targeting a sequence of glutamate gene dehydrogenase (gdh), and only 9 were positive (10.7%) to the nested PCR targeting the 18S rRNA gene [7].

Thus, the sensitivity of real-time PCR in our study was in line with those of previous studies. We showed an overall sensitivity of 76.6% ranging from 58.3% in the school of Linoghin A to 94.1% in the school of Doure A. The overall specificity of the real-time PCR was 96.2% ranging from 95.4% in the Centre-Ouest region to 96.7% in Plateau Central region.

We also found two real-time positive cases among the 53 microscopy-negative stool samples. Here, considering microscopy as 'gold' standard, these unmatched cases can be considered as false positives. It is possible that we have overestimated the microscopy-positive cases of *G. intestinalis* in our study, including potential false positives.

The lack of PCR detection of the 22 microscopy-positive samples suggests an absence of target DNA and could be explained by lysis of cysts followed by a possible degradation of the parasite DNA by the DNase contained in faeces during sample storage [33]. Indeed, no preservatives have been added to the samples during the storage period while keeping the samples at -20°C in contrast to other study protocols [33,34]. In addition, PCR-based DNA amplification can be inhibited by the decomposition products of haemoglobin in stool such as bilirubin, bile acids and inorganic ions [33,35]. In our study, we have not provided special purification cysts prior to DNA extraction, while other authors advocate such a procedure [36]. Indeed, in such a context where the likely rate of PCR inhibition is high (23.4%), the optimization of the DNA extraction protocol to eliminate inhibitors was shown to increase the yield [30,37]. However, the low concentration of *G. intestinalis* in stool samples could also be a reason for non-amplification. Even if real-time PCR can detect a single cyst [29], it is still necessary that the cyst is contained in the 0.1 g of stool sample collected for DNA extraction. Sometimes, we have identified only a few cysts of *G. intestinalis* but only after the concentration of the stool by the formalin-ether technique. Regarding the real-time PCR on *G. intestinalis*, Bertrand et al. showed that for an initial concentration of 180 cysts per 200 μl of preparation, only 33% of positive responses were observed [38,39]. Indeed, a relatively low sensitivity (93.4%) was observed by targeting sequences of the small subunit gene of rRNA [40] as in our study.

PCR has major benefits to significantly increase the speed, the sensitivity and specificity for detection of microorganisms. It is, however, sensitive to a large number of inhibitory compounds which can lead to an underestimation of cysts and false negative results.

Conclusion

For the first time in Burkina Faso, real-time PCR, not yet routinely available in African laboratories, was used to detect *G. intestinalis* from cysts. Our data show that real-time PCR can be of great help in the diagnosis of giardiasis. However, it needs to be further optimized to become a more sensitive tool for *G. intestinalis* diagnosis in low-income settings.

We found a strong association between infection of *G. intestinalis* and *E. histolytica/E. dispar*. In future studies, it would be interesting to use a multiplex PCR to simultaneously detect these intestinal protozoa on the same sample.

Conflict of Interest

None declared

Authors Contributions

SD, TK, SE, PO, JU, GC and JS conceived the study. TK, TRC, AZ, GT and BS performed the experiments. SD, JS and GC contributed for reagents and materials of the experiments. SD, TK, SE and JS analysed the data and wrote the manuscript. All authors reviewed drafts and approved the final version of the manuscript.

Acknowledgements

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