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Real-time multiplex PCR diagnosis of common diarrhoea-causing parasites in children under five years in Ouagadougou, Burkina Faso

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ABSTRACT:

Background: *Giardia intestinalis, Cryptosporidium spp* and *Entamoeba histolytica* are obligate pathogens and are the most common causative agents of childhood diarrhoea. The diagnosis of these parasitic infections was for a long time based on microscopic examination of stools that lacks sensitivity and specificity and requires a highly trained staff. As a result of these limitations, DNA-based detection methods exhibiting numerous advantages such as increased sensitivity and specificity, ability to combine multiple targets in one multiplex assay have been developed for enteric parasites.
This study aimed to assess the epidemiology of these three-common diarrhoea-causing parasites in children under five years using microscopy vs. real time PCR assay.

Patients and Methods: A hospital-based cross-sectional survey for gastrointestinal parasites in children under five years was conducted in Ouagadougou from January 2018 to December 2019. Stool samples from 188 children presented with diarrhoea were processed using both light microscopy and real time PCR techniques.

Results: Microscopy showed a 22.9% (43/188) overall prevalence of parasitosis with 10.1% (19/188) *E. histolytica*/*E.* dispar and 6.9% (13/188) *G. intestinalis.* Real time PCR was positive in 26.1% (49/188) of cases. *G. intestinalis* was the most common with a 22.9% (43/188) prevalence followed by *Cryptosporidium spp* 6.4% (12/188) and *E. histolytica* 1.6% (3/188).

Conclusions: Intestinal protozoan parasites mainly affect children aged 12-24 months. *G. intestinalis* was identified as the leading cause of childhood diarrhoea. The real time PCR assay showed an excellent sensitivity detecting gastrointestinal parasites comparatively to microscopy that exhibited false positive or negative cases.

Keywords: Children, Diarrhoea, Protozoan parasites, Real time PCR, Burkina Faso.

Abbreviations: AIDS: Acquired Immune Deficiency Syndrome; B. : Blastocistis; DNA: Deoxyribonucleic acid; DNA-J: J domain protein Deoxyribonucleic acid; E. : Entamoeba; G. : Giardia; HIV: Human Immunodeficiency Virus; PCR: Polymerase Chain Reaction; RNA: Ribonucleic acid; SSU rRNA: Small Subunit ribosomal Ribonucleic acid; T. : Trichomonas.

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BACKGROUND

Parasitic gastroenteritis remains a major public health concern in the tropics due to favourable climatic conditions, poor hygiene and sanitation and low socio-economic status[1,2]. Parasitic gastroenteritis, considered a disease of poverty, is of little interest nowadays and affects most the developing world, particularly in sub-Saharan Africa, Asia and Latin America[1,3,4]. Protozoan parasites can infect the human intestinal tract causing serious diseases. *Giardia intestinalis*, *Cryptosporidium spp* and *Entamoeba histolytica* are obligate pathogens and are the most common causative agents of persistent childhood diarrhoea[5]. They are the leading causes of childhood morbidity and mortality worldwide[1,6] and they can lead to malnutrition, anaemia, decreased resistance to infection, diarrhoea and stunting[7-10].

Traditionally, intestinal protozoa are routinely detected by light microscopy which has the advantages of being largely affordable, relatively easy to perform in resource-limited settings, and able to detect several parasites of clinical significance. Most intestinal protozoa undergo different life stadia within the gastrointestinal tract, resulting in visibility of a cyst and/or trophozoite stage on microscopy. Microscopic detection and differentiation of different protozoa are based on small but specific morphological differences in cyst and trophozoite stage, but these distinctive morphologies are often difficult to distinguish. Also, detection of particular protozoa requires specific staining methods[11,12]. The microscopic examination of feces is laborious and requires specifically trained staff. Light microscopy testing also suffers from low sensitivity and specificity that may result in an inadequate diagnosis of intestinal protozoa[12-16]. Therefore, real time PCR is now emerging as a powerful tool in the routine detection, quantification and typing of intestinal parasitic protozoa. The main advantages of real time PCR are that it provides fast and high-throughput detection and quantification of target DNA sequences, a lower time of amplification facilitated by the simultaneous monitoring of newly formed DNA amplicons. Moreover, real time PCR is safer in terms of avoiding cross contaminations as no further post-PCR handling is required. Other advantages of real time PCR include a wide dynamic range for quantification and the multiplexing of amplification of several targets into a single reaction[15,17,18]. Real time PCR also is of high sensitivity and specificity but its use remains limited due to the technical complexity, the very high cost of reagents and equipment and the time required to perform the analysis compared to traditional light microscopy technique.

Burkina Faso faces this endemic parasitic gastroenteritis. However, there is scarcity of data on the epidemiology of parasitic diseases[19]. In this study, both microscopy and real-time PCR were used in a comparative fashion to determine the prevalence of several intestinal protozoan parasites. This study aimed to re-evaluate the epidemiology of these protozoan parasites in children under five years of age using molecular techniques for a better management of patients and for the development of integrated and effective control strategies.

PATIENTS AND METHODS

Patients and study setting

A descriptive cross-sectional study to diagnose parasitic gastroenteritis in children under 5 years old was performed at Pietro Annigoni Biomolecular Research Centre/Laboratory of Molecular Biology and Genetics (CERBA/LABIOGENE). The study was conducted from January 2018 to December 2019 and included 188 consecutive children under 5 years old from the city of Ouagadougou. Stool samples were collected from patients with diarrhoea seeking medical care. A parasitological examination of stool specimens was requested by a clinician during the study period.

Stool collection and processing

The stools of the patients, freshly emitted, were collected in a sterile plastic container and sent directly to the medical parasitology lab of Pietro Annigoni Biomolecular Research Centre to be examined within one hour of their emission. The fresh faecal samples were then processed by direct wet smear followed by iodine staining and examined via microscopy for the presence of intestinal parasites. The Motic BA300 Binocular Compound Microscope (Motic Instruments, Canada) was used for analysis. The fresh stools were then aliquoted in 2.5 mL sterile cryotubes and were stored at –20°C and further assessed using real-time PCR technique.

DNA isolation

Genomic DNA was extracted from faecal samples using QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the protocol provided by the manufacturer. Prior to DNA extraction, stool samples were first thawed at room temperature and pre-treated as follows: 200 µL (≈200 µg) stool samples were collected and dispensed into a 2 mL Eppendorf (EP) tube. Then, 800 µL of phosphate-buffered saline (PBS) was added. The mixture was vortexed vigorously for 1 min and incubated at 80°C for 10 min. After incubation, the mixture was vortexed for 15 s and centrifuged at 14000 rpm for 1 min. Two hundred (200) µL of the supernatant were then recovered and transferred to a new 1.5 mL Eppendorf tube and the protocol was followed as per the manufacturer's instructions. A lysis solution was prepared by adding to the lysis buffer 2 µL internal control for each sample according to the FTlyo Stool Parasites Kit (Fast Track Diagnostics, Luxembourg) instructions. The purity and the final concentration of the DNA extracts were determined using the Biodrop µLITE spectrophotometer (Isogen Life Science N.V./S.A, Temse, Belgium). The extracted DNA was stored at -20°C up to the amplification step.

Real time PCR assay

The real time PCR assay to identify *Entamoeba histolytica*, *Giardia intestinalis* and *Cryptosporidium spp*

Table 1. Sociodemographic characteristics of patients.

		N	%
Gender	Male	101	53.7
	Female	87	46.3
Age group	2-11 months	24	12.8
	12-24 months	102	54.2
	25-60 months	62	33.0

was carried out using the FTlyo Stool Parasites kit (Fast Track Diagnostics, Luxembourg) according to manufacturer's instructions. This multiplex assay is targeting the SSU rRNA gene sequences for *Entamoeba histolytica* and *Giardia intestinalis* and the DNA-J like protein gene for *Cryptosporidium spp*[20]. Briefly, 15 µL of extracted DNA and controls were added to the lyophilized master mix in an 8-well strip. The strips were then sealed and centrifuged at 2500 rpm for 2 minutes before being introduced into the 7500 Fast real time PCR system (Applied Biosystems, Foster City, CA, USA) for amplification. The cycling conditions were: 50°C for 15-min hold, 94°C for 1-min hold followed by 40 cycles consisting: 94°C for 8 sec and 60°C for 1 min. A cooling stage of +4°C for 2 min was added at the end of the amplification. Fluorescence detection was performed using the TaqMan technology (Applied Biosystems™ TaqMan® chemistry, Foster City, CA, USA) using the following detection channels: *Entamoeba histolytica* was detected by "JOE", *Giardia intestinalis* by "Cy5", *Cryptosporidium spp* by "Texas Red" and the internal control (*Streptococcus equi*) by "FAM". The results were analysed on computer using 7500 Fast software_v2.0.6 (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

The data was entered on Microsoft Excel 2019 and analysed using the Standard Statistical Package for Social Sciences (SPSS) version 20.0 (IBM, Armonk, NY, USA). Study participants were subdivided into 3 age groups for analysis: 2 to 11 months, 12 to 24 months, 25 to 60 months. The qualitative variables were described in terms of size and percentage of data.

Ethics

The Burkina Faso' National Health Research Ethics Committee approved this study (Approval Decision No. 2018-11-141). The study was explained to the parents or legal guardians of the children and informed consent was confirmed by signature or fingerprint if illiterate prior to enrolment in the cohort. The investigator recorded clinical and demographic information on a standard form. Parasitological examination was performed free of charge and results were immediately transmitted to the clinician for patient's management.

RESULTS

Characteristics of the study population

A total of 188 children aged 2-60 months presented with diarrhoea were enrolled. Diarrhoea has been defined as the emission of at least 3 liquid or soft stools per day. This study population consisted mostly of 54.2% (102/188) children aged from 12-24 months. Male patients accounted for 53.7 % (101/188) of cases and sex ratio was 1.15 (Table 1).

Prevalence of gastrointestinal parasitic infections given by light microscopy

The overall prevalence of parasitosis was 22.9% (43/188). The parasites encountered during the study period were: *E. histolytica/E. dispar*, *E. coli*, *G. intestinalis*, *T. intestinalis* and *B. hominis* with respective prevalence of 10.1% (19/188), 6.9% (13/188), 6.9% (13/188), 1% (2/188) and 0.5% (1/188). Children aged 25-60 months presented 52.1% (25/48) of parasitosis followed by those aged 12-24 months with 41.7% (20/48) (Table 2).

Prevalence of diarrhoea-causing parasites given by real-time multiplex PCR

Molecular diagnosis by real-time multiplex PCR for *E. histolytica*, *G. intestinalis* and *Cryptosporidium spp* was positive in 26.1% (49/188) of cases. Parasitic coinfections were recorded at a rate of 16.3% (8/49) (Table 3). *G. intes-*

Table 2. Age distribution of parasites detected by light microscopy.

	Age group (months)			
Parasites	**2-11 N (%)**	**12-24 N (%)**	**25-60 N (%)**	**Total N (%)**
E. histolytica/E. dispar	1 (5.3)	5 (26.3)	13 (68.4)	19 (100)
Entamoeba coli	1 (7.7)	7 (53.8)	5 (38.5)	13 (100)
Giardia intestinalis	1 (7.7)	6 (46.2)	6 (46.2)	13 (100)
Trichomonas intestinalis	0 (0)	2 (100)	0 (0)	2 (100)
Blastocystis hominis	0 (0)	0 (0)	1 (100)	1 (100)
Total	3 (6.3)	20 (41.7)	25 (52.0)	48 (100)

Table 3. Raw real-time multiplex PCR results.

Parasites	N	%
Giardia intestinalis	37	19.7
Cryptosporidium spp	4	2.1
Cryptosporidium spp and *Giardia intestinalis*	5	2.7
Cryptosporidium spp and *Entamoeba histolytica*	2	1.1
Cryptosporidium spp and *Entamoeba histolytica* and *Giardia intestinalis*	1	0.5
Negative	139	73.9

Table 4. Prevalence of diarrhoea-causing parasites by real-time multiplex PCR.

Parasites	N	%
Giardia intestinalis	43	22.9
Cryptosporidium spp	12	6.4
Entamoeba histolytica	3	1.6

tinalis was the most common parasite in children with a prevalence of 22.9% (43/188) (Table 4). *G. intestinalis* and *Cryptosporidium spp* primarily affected children aged from 12-24 months, who accounted for 56.9% (33/58) of the parasitic infection cases (Table 5).

Comparison for microscopy *vs.* real-time PCR results

Real-time PCR assay was performed on all samples tested by light microscopy and 80.7% (117/145) of the microscopically negative samples remained negative to real-time PCR. The microscopically negative samples left (28/145) were positive for either *G. intestinalis*, *Cryptosporidium spp* and *E. histolytica* alone or co-infected. Eighty-four point six (84.6%) (11/13) of the microscopically positive samples for *G. intestinalis* were positive for *G. intestinalis* and/or *Cryptosporidium spp* on real-time PCR. Thirty-eight point five (38.5%) (5/13) of the *E. coli* microscopically positive samples were positive for *G. intestinalis* and/or *Cryptosporidium spp* in real-time PCR. Out of the microscopically positive samples for *E. histolytica/E. dispar*, 68.4% (13/19) remained negative and 31.6% (6/19) were positive *for G. intestinalis* and/or *Cryptosporidium spp* in real-time PCR. None of the microscopically positive samples for *E. histolytica/E. dispar* were positive for *E. histolytica* in real-time PCR. The *E. histolytica* positive samples in real time PCR were recorded within the microscopy negative samples. Light microscopy assay was negative for *Cryptosporidium spp* (Table 6).

DISCUSSION

Cryptosporidium spp, E. histolytica and *G. intestinalis* are the main diarrhoea-causing protozoa worldwide. Infections caused by these parasites are major sources of morbidity and mortality mainly among children living in resource-limited countries[21-25]. Accurate diagnosis of the etiological agents of acute diarrhoea is therefore of great importance for the management of diarrhoeal diseases, which can be treated symptomatically or with a wide range of antiparasitic drugs[5,26-31]. Despite recent advances in diagnostic technology, microscopic examination of stool specimens remains the cornerstone of diagnostic testing for most pathogenic intestinal protozoa. Microscopy is still the gold standard technique for the laboratory detection of gastrointestinal parasites[11,16]. Here we used a light microscopy assay to identify gastrointestinal parasites in children under 5 years age in Burkina Faso. *E. histolytica/E. dispar* and *G. intestinalis* were the most common parasites isolated in the stools of children with diarrhoea. These parasites infect intestinal epithelial cells and release proteins via micro and macrovesicles degrading tight junction proteins and villin to disrupt epithelial barrier and induce diarrhoea[5,32,33]. *E. histolytica/E. dispar* and *G. intestinalis* occurred predominantly in children aged 25-60 months. The faeco-oral transmission of these parasites via contaminated food or drinking water explains its strong presence in children over 2 years of age. Indeed, poor food hygiene at the time of dietary diversification could favour parasitic contamination[19].

Table 5. Age distribution of diarrhoea-causing parasites by real-time multiplex PCR.

	Age group (months)			
Parasites	2-11 N (%)	12-24 N (%)	25-60 N (%)	Total N (%)
Giardia intestinalis	4 (9.3)	25 (58.1)	14 (32.6)	43 (100)
Cryptosporidium spp	2 (16.7)	7 (58.3)	3 (25)	12 (100)
Entamoeba histolytica	0 (0)	1 (33.3)	2 (66.7)	3 (100)
Total	06 (10.3)	33 (56.9)	19 (32.8)	58 (100)

Table 6. Results comparison of light microscopy over real-time multiplex PCR.

	Real-time multiplex PCR				
	Negative	***Giardia intestinalis***	***Cryptosporidium spp***	***Entamoeba histolytica***	**Total**
Light microscopy					
Negative	117	24	5	3	149[1]
Giardia intestinalis	2	9	3	0	14[2]
Entamoeba histolytica/ Entamoeba dispar	13	6	1	0	20[3]
Trichomonas intestinalis	2	0	0	0	2
Entamoeba coli	8	4	3	0	15[4]
Blastocystis hominis	1	0	0	0	1
Total	143[5]	43	12	3	

[1]Four microscopically negative samples were positive for *E. histolytica* and *Cryptosporidium spp*, *G. intestinalis* and *E. histolytica* and *Cryptosporidium spp*, *E. histolytica* and *Cryptosporidium spp*, *G. intestinalis* and *Cryptosporidium spp*, respectively, resulting in a total of 149 instead of 145.
[2]A sample positive for *G. intestinalis* on microscopy was positive for *G. intestinalis* and *Cryptosporidium spp* on PCR resulting in a total of 14 instead of 13.
[3]A sample positive for *E. histolytica/E. dispar* on microscopy was positive for *G. intestinalis* and *Cryptosporidium spp* on PCR resulting in a total of 20 instead of 19.
[4]Two microscopically positive samples for *E. coli* were positive for *G. intestinalis* and *Cryptosporidium spp* in PCR resulting in a total of 15 instead of 13.
[5]Two PCR negative samples were positive for *E. histolytica/E. dispar* and *G. intestinalis, E. coli* and *G. intestinalis* and *T. intestinalis microscopically*, resulting in a total of 143 instead of 139.

Since microscopy presents a limit in low parasites individual or mixt infections, real time PCR is a useful tool in this setting. Stool specimens were then analysed using a real time multiplex PCR assay for *E. histolytica*, *G. intestinalis* and *Cryptosporidium spp.* The results revealed that *G. intestinalis* was the most prevalent (22.9%) in our study population. This data is definitely confirming in agreement with previous studies that *G. intestinalis* is the leading cause of childhood parasitic gastroenteritis in Burkina Faso using both microscopy or PCR techniques[19,34-38]. *Cryptosporidium spp* was the second most prevalent (6.4%) parasite in children with diarrhoea. Cryptosporidium infection causes tissue damage occurring through disruption of tight junctions of the epithelium due to the release of lytic enzymes, leading to cytoskeletal alterations, loss of barrier function and ultimately enhancing the symptoms like malabsorption and diarrhoea[5,39,40]. Cryptosporidium is known as a causative agent of persistent diarrhoea that can be life-threatening for people with weakened immune systems, such as people with HIV/AIDS. We were unable to explore the nutritional and HIV status of these children to determine whether their cryptosporidiosis was opportunistic. The link between malnutrition and cryptosporidiosis has often been discussed but remains difficult to establish. Nevertheless, two types of malnutrition-cryptosporidiosis link can be envisaged: cryptosporidiosis responsible for malnutrition through persistent diarrhoea or conversely, malnutrition responsible for a weakening of the immune defences that maintain cryptosporidiosis[41-43]. It was the first time in Burkina Faso, Cryptosporidium is identified in stool specimens using molecular technique. As Cryptosporidium was not diagnosed using microscopy, our data are highlighting the need of specific staining for cryptosporidium that is not systematically performed in routine laboratory testing for gastrointestinal pathogens. Like all coccidian intestinal parasites, the small and poorly staining Cryptosporidium oocysts can be easily missed in routine stool parasites testing. Indeed, sensitivity of light microscopy for Cryptosporidium is improved by performing modified acid-fast (MAF) stains[11,16].

We aimed to compare the diagnostic accuracy of light microscopy to real time PCR. High prevalence of *G. intestinalis* was found in this study using both microscopy and PCR. Its highly distinctive morphology facilitates microscopic diagnosis. Giardia cysts or trophozoites can be easily observed in fresh smears, or permanent stained smear[11]. The real time PCR assay highly increased (6.9% from microscopy to 22.9% by PCR) the detection rate of *G. intestinalis* indicating the higher sensitivity of this technique for a parasite with irregular shedding. Moreover, low grade infections would be missed by microscopy because identification depends on individual experience and skills[44]. The diagnosis of *E. histolytica* showed intriguing results as no *E. histolytica/E. dispar* microscopically positives samples were positive for *E. histolytica* using real time PCR. Considering that *E. histolytica* and *E. dispar* cannot be differentiated morphologically, the cases detected by microscopy were reported as *E. histolytica/E. dispar.* All the *E. histolytica/E. dispar* cases (13/19) negative for *E. histolytica* in real time PCR should be considered as *E. dispar* infections although specific PCR test for *E. dispar* is performed. Zongo et al[5] stated the relatively low prevalence of *E. histolytica* in genus Entamoeba infections in Burkina Faso. In addition, we may think

of the possible genetic variability of the *E. histolytica* circulating strains in the country. Suzuki et al[46] reported an *E. histolytica* novel strain (BF-841 cl1) that originated from Burkina Faso, presented with polymorphic genotypes of the serine-rich *E. histolytica* protein and the hexokinase-2 gene than that of an *E. histolytica* reference strain [HM-1:IMSS]. It could be necessary to use sequencing to definitely address this discrepant finding in our study. This emphasises the need to better diagnose this parasite species, differentiating it from the non-pathogenic *E. dispar* using other methods than morphology because many infected people are being treated purposeless.

CONCLUSIONS

This study demonstrates that parasitic gastroenteritis is still a public health concern in Burkina Faso with high prevalence of gastrointestinal parasites despite improved hygiene conditions. Intestinal protozoan parasites mainly affect children aged 12-24 months. *G. intestinalis* was identified as the leading cause of childhood diarrhoea. The real time PCR showed an excellent sensitivity detecting gastrointestinal parasites comparatively to microscopy that exhibited false positive or negative cases. Real time PCR should be considered the gold standard technique for the diagnosis of parasitic gastroenteritis.

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Conflict of Interest:
The authors declare that they have no conflict of interest.

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