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Performance of Real-Time Polymerase Chain Reaction Assays for the Detection of 20 Gastrointestinal Parasites in Clinical Samples from Senegal

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Abstract. Gastrointestinal parasite infections represent one of the biggest public health problems in the world. Therefore, appropriate innovative tools are needed for assessing interventions to control these infections. This study aims to compare the performance of real-time polymerase chain reaction (PCR) assays to microscopic examination for detection of intestinal parasites. A direct microscopic examination and stool concentration was performed on 98 stool samples from patients attending Senegalese hospitals. Negative microscopic control samples were also collected in Nice and Marseille (France). Species-specific primers/probes were used to detect 20 common gastrointestinal protozoans and helminths. Positive frequency and the sensitivity of each real-time PCR assay were compared with conventional microscopic examination. Real-time PCR was positive in 72 of 98 samples (73.5%), whereas microscopic examination was positive in 37 (37.7%) samples ($P < 0.001$). The real-time PCR assays were more sensitive than microscopy, with 57.4% (31/54) versus 18.5% (10/54), respectively, in the detection of parasites in asymptomatic patients ($P < 0.05$). In terms of polyparasitism, there were more coinfections detected by real-time PCR assays compared with microscopic methods (25.5% versus 3.06%). In comparison to parasite prevalence on individual samples, the results showed a perfect agreement (100%) between the two techniques for seven species, whereas discrepancies were observed for the others (agreement percentage varying from 64.2% to 98.9%). Real-time PCR appeared to be superior to microscopic examination for the detection of parasites in stool samples. This assay will be useful in diagnostic laboratories and in the field for evaluating the efficacy of mass drug administration programs.

INTRODUCTION

Infections due to gastrointestinal parasites represent one of the biggest public health problems in the world. According to the World Health Organization, more than 1 billion people are infected with nematodes that cause soil-transmitted helminthiases.[1] Helminth and protozoan infections play major roles in the occurrence of the main digestive disorders causing morbidity and mortality worldwide.[2] Among other problems, anemia, malnutrition, and gastrointestinal complaints, particularly diarrhea, are associated with these infections. Indeed, diarrheal diseases were responsible for more than 1.4 million deaths in 2010, ranking it the seventh leading cause of death to which children are most vulnerable.[3] Unfortunately, these intestinal parasitic diseases are underestimated in limited-resource settings, particularly in Africa, due to the lack of sensitive and accurate diagnostic tools.

Microscopic examination of stool samples is the most widely used diagnostic approach for intestinal parasitic detection. First, direct microscopic examination is performed by mixing a small amount of feces with physiological sodium chloride solution (0.9%). Then, various stool concentration techniques based on the use of either sedimentation or flotation with a formalin-ether concentration technique are performed to increase sensitivity.[4–6] Microscopic examination is not expensive and is able to screen for a maximum of parasites in one test, whereas molecular detection is limited to the targeted species. However, this microscopic diagnostic method lacks sensitivity and reproducibility, particularly in epidemiological investigation, and cannot distinguish species of some parasites based on their eggs such as *Ancylostoma duodenale*/*Necator americanus* or *Taenia saginata*/*Taenia solium*. Moreover, accurate diagnosis with microscopy depends on the experience of the laboratory's microscopist and the concentration of parasite material in the sample. Finally, some parasite species such as *Entamoeba histolytica*, *Cryptosporidium* sp., and *Strongyloides stercoralis*, which are responsible for severe infections, are often misdiagnosed even when concentration techniques are used.[7,8]

To overcome these deficiencies, molecular techniques have been suggested as a complementary process and may be an alternative to microscopic examination. Indeed, conventional and real-time polymerase chain reaction (PCR) have proven to be sensitive and accurate for helminth and intestinal protozoan detection.[9] These techniques have the advantage of detecting low parasite levels, improving the identification of infected persons, and assessing treatment effects by quantification.[10] Moreover, a technician trained in PCR could run multiple tests to detect different classes of pathogens such as viruses, bacteria, and parasites. Real-time PCR is more attractive compared with conventional PCR, as the methodology reduces the risk of contamination and decreases the cost of reagents.[8,11] To date, several real-time PCR assays have been developed separately to detect common helminths and intestinal protozoans.[9] However, most of the studies assessing real-time PCR are limited to a small number of species.[8,10,12]

Given the need to control emerging and neglected tropical diseases, it is important to have innovative tools such as

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real-time PCR for accurate diagnoses. To do so, we need to assess the performance of real-time PCR assay in the detection of a maximum of intestinal parasites with the same protocols to avoid interlaboratory variations. That is why we have performed this study to compare the real-time PCR assay to microscopic examination for the detection of 20 gastrointestinal parasites in the same laboratory.

MATERIALS AND METHODS

Sample collection. One hundred and three samples were collected between August and November 2014 from patients with or without abdominal symptoms in three hospitals (Fann teaching hospital, Roi Baudouin hospital of Guédiawaye and Dagana health center) in Senegal (a west African country). Feces were collected in appropriately sealed, labeled, and clean pots. The samples were collected in the laboratory, so the reading was done immediately or within 30 minutes. The stools received from hospitalized patients were transported on ice and examined within 1 hour. They were divided into two groups: positive and negative according to microscopy results. All slides were examined by two experienced microscopists. Due to discrepancies between the two reads, five specimens were removed from the analysis process. At the end, 98 fecal samples were further used for the study. After microscopic examination, all samples were fixed in absolute ethanol (96–100%) immediately after the concentration step, typically between 45 minutes and 1 hour after collection, and stored at 4°C before transportation to Marseille for molecular testing. Sociodemographic data (e.g., age, sex, and symptoms) for each sampled individual were also collected. Ninety-four microscopy-negative stool samples were collected as control in France, including 48 from Nice (Center Hospitalier Universitaire) and 46 from Marseille (Laboratoire Alphabio of Hôpital Européen). Stool samples were obtained from patients with or without symptoms, and who were received in these hospitals for parasitic infection diagnosis. Only specimens with negative microscopy results (no parasites identified after two readings by two microscopists) were tested by real-time PCR and compared with negative microscopy results from Senegal. The objective was to assess the performance of real-time PCR to detect parasites missed by microscopy in tropical and nontropical regions.

Microscopic examination. Each sample collected in Senegal was first examined by direct saline solution, iodine mounts, and after concentration by the formol-ethyl acetate technique. Modified Ziehl–Neelsen staining was performed on direct fresh smears and on formol-ethyl acetate concentrates to detect *Cryptosporidium*, *Cyclospora*, and *Cystoisospora* species. For the identification of microsporidial spores, smears were prepared using concentrated sediment and stained with the modified trichrome method. A sample was considered negative if no parasite was identified after the examination of all fields on the prepared slide by the two readers. Samples from France were tested first in direct saline solution and after concentration with the routine method used by each laboratory. Trichrome staining was not performed in Marseille and Nice.

DNA extraction. DNA was extracted from stool samples using a modified method of the Qiagen stool procedure (QIamp DNA Stool Mini Kit, Qiagen, Courtaboeuf, France).[13,14] Aliquots of 200 mg (200 µL for liquid/diarrheic stools) of stool sample were placed in 2-mL tubes containing 200 mg of 2-mm glass beads and 1.5 mL of a stool lysis (ASL) buffer (Qiagen). The samples were mixed vigorously by agitation in a FastPrep BIO 101 agitator (Qbiogene, Strasbourg, France) at 3,200 rpm for 90 seconds, followed by heating at 95°C for 10 minutes. The final pellet was suspended in 200 µL of tissue lysis buffer and incubated with 30 µL of proteinase K for 2 hours at 55°C. Then, the manufacturer's recommendations were followed for the purification and elution of the DNA. Inhibition was assessed for each sample by addition of an exogenous synthetic oligonucleotide and an internal control that was extracted and amplified. First, 70 µL of a synthetic sequence of 142 bp (5′-GCTACTGAGTCGTACCTAATGCATGACCTAGAGCAC TCGACTGTTTATCAGTGTCGAGACTCGACGCATGCACGTA CGAACCTAGCTGTCAGCAATCGCGAATGCCTACTAAGT AGCGAACTTTAGCGAATCGCGATACGAC-3′) routinely used in the laboratory and ordered at 200 nmol was diluted at 10^7 and was added in the tube containing the stool sample and the ASL buffer. The sequence was then amplified at the end of the extraction process in a real-time PCR assay by a set of primers (TissF_5′-CTGAGTCGTACCTAATGCATGACC-3′; TissR_5′-GTATCGCGATTCGCTAAAGTTC-3′) and probe (TissP_6FAM-5′-TCGAGACTCGACGCATGCACG-Tamra-3′). A second internal control consisting in the amplification of all bacteria in the stool sample by a simplex real-time PCR was used as described in the literature.[15]

Singleplex real-time PCR amplification and detection. Twenty different specific primers and Taqman™ probes (hydrolysis probes) targeting sequences regions were used in multiparallel assays, including 17 published and three newly designed ones, as shown in Table 1. Specific primers/probes used for the first time in this study were designed using multiple sequence alignment ClustalW2 (EMBL-EBI, Cambridgeshire, UK) and the PRIMER 3 (Rozen S, Singapore) software. The specificity of each primer was tested using the basic local alignment search tool, available at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/).

All primers and probes used in the study were assessed for analytical sensitivity and specificity. To determine the limit of detection, plasmids with each specific target sequence were diluted to a fixed concentration and serially diluted to a final concentration of 10 copies/5 µL. For the analytical specificity, each species-specific assay was tested against other parasite DNA preparations to detect any cross-reactivity.

The real-time PCR reactions were conducted using 20 µL total volumes containing 10 µL of master mix (Quantitect; Qiagen), 0.5 µL of each primer (20 µM), 2 µL of probes (3 µM), 2 µL of distilled water, and 5 µL of template DNA. Analyses were performed using a CFX96™ Real-Time PCR detection assay (Bio-Rad Life Science, Marnes-la-Coquette, France). Amplification reactions were done as follows: 95°C for 15 minutes followed by 44 cycles of 60°C for 0.5 minutes and 72°C for 1 minute. Positive (parasite-specific oligonucleotides) and negative controls were tested in each run.

The real-time PCR assays were carried out in duplicate for reproducibility. During the first assay, samples were run without using positive control to avoid any possible contamination with the plasmid DNA. In a second assay, all samples including positive and negative ones from the first run were tested again with plasmid control DNA launched in

TABLE 1

List of primers and probes (Applied Biosystems, Cheshire, UK) used in this study for the detection of 20 intestinal parasites by real-time PCR

Parasites	Name	Primers/Probes	Target region	Reference
Protozoa				
Balantidium coli	BcoliF	5′-TGCAATGTGAATTGCAGAACC-3′	ITS-1	Designed in this study
	BcoliR	5′-TGGTTACGCACACTGAAACAA-3′		
	BcoliP	5′-FAM-CTGGTTTAGCCAGTGCCAGTTGC-TAMRA-3′		
Blastocystis spp.	Blasto FWD F5	5′-GGTCCGGTGAACACTTTGGATTT-3′	SSU rRNA gene	16
	Blasto R F2	5′-CCTACGGAAACCTTGTTACGACTTCA-3′ 5′-FAM-		
	Blasto probe	TCGTGTAAATCTTACCATTTAGAGGA-MGBNFQ-3′		
Cryptosporidium parvum; *C. hominis*	1PSF	5′-AACTTTAGCTCCAGTTGAGAAAGTACTC-3′	hsp70 gene	17
	1PSR	5′-CATGGCTCTTTACCGTTAAAGAATTCC-3′		
	cryptP	5′-FAM-AATACGTGTAGAACCACCAACCAATA CAACATC-TAMRA-3′		
Cyclospora cayetanensis	Cyclo250F	5′-TAGTAACCGAACGGATCGCATT-3′	18s	18
	Cyclo350R	5′-AATGCCACGTAGGCCAATA-3′		
	Cyclo281T	5′-FAM-CCGGCGATAGATCATTCAAGTTTCTGACC-TAMRA-3′		
Dientamoeba fragilis	Df-124F	5′-CAACGGATGTCTTGGCTCTTA-3′	18S	19
	Df-221R	5′-TGCATTCAAAGATCGAACTTATCAC-3′	18S	
	Df-172revT	5′-FAM-CAATTCTAGCCGCTTAT-MGB-3′	18S	
Enterocytozoon bieneusi	FEB1	5′-CGCTGTAGTTCCTGCAGTAAACTATGCC-3′	18s	20
	REB1	5′-CTTGCGAGCGTACTATCCCCAGAG-3′		
	PEB1	5′-FAM-ACGTGGGCGGGAGAAATCTTTAGTG TTCGGG-TAMRA-3′		
Encephalitozoon intestinalis	FEI1	5′-GCAAGGGAGGAATGGAACAGAACAG-3′	18s	21
	REI1	5′-CACGTTCAGAAGCCCATTACACAGC-3′		
	PEI1	5′-FAM-CGGGCGGCACGCGCACTACGATA-TAMRA-3′		
Entamoeba histolytica	Ehf	5′-AACAGTAATAGTTTCTTTGGTTAGTAAAA-3′	18s	22
	Ehr	5′-CTTAGAATGTCATTTCTCAATTCAT-3′		
	Ehp	5′-FAM-ATTAGTACAAAATGGCCAATTCATTCA-TAMRA-3′		
Giardia lamblia (*intestinalis* or *duodenalis*)	*Giardia*-80F	5′-GACGGCTCAGGACAACGGTT-3′	18s	23
	Giardia-127R	5′-TTGCCAGCGGTGTCCG-3′		
	Giardia-105T	5′-FAM-CCCGCGGCGGTCCCTGCTAG-TAMRA-3′		
Cystoisospora belli	Ib-40F	5′-ATATTCCCTGCAGCATGTCTGTTT-3′	ITS2	24
	Ib-129R	5′-CCACACGCGTATTCCAGAGA-3′		
	Ib-81Taq	5′-FAM-CAAGTTCTGCTCACGCGCTTCTGG-TAMRA-3′		
Helminths				
Ancylostoma duodenale	Ad125F	5′-GAATGACAGCAAACTCGTTGTTG-3′	ITS2	25
	Ad195R	5′-ATACTAGCCACTGCCGAAACGT-3′		
	Ad155-XS	5′-FAM-ATCGTTTACCGACTTTAG-MGB-3′		
Ascaris lumbricoides	Alum96F	5′-GTAATAGCAGTCGGCGGTTTCTT-3′	ITS-1	26
	Alum183R	5′-GCCCAACATGCCACCTATTC-3′		
	Alum124T	5′-FAM-TTGGCGGACAATTGCATGCGAT-TAMRA-3′		
Hymenolepis diminuta	HymF	5′-GTTGTATCAGGGAGTGGTG-3′	ITS-1	Designed in this study
	HymR	5′-AATTCACATCTCGTGCCTTG-3′		
	HymP	5′-FAM-TGCTGCAGTTCACTAACCGTGGC-TAMRA-3′		
Necator americanus	Na58F	5′-CTGTTTGTCGAACGGTACTTGC-3′	ITS-2	25
	Na158R	5′-ATAACAGCGTGCACATGTTGC-3′		

(continued)

TABLE 1
Continued

Parasites	Name	Primers/Probes	Target region	Reference
Schistosoma mansoni	Na81Tmgb	5′-FAM-CTGTACTACGCATTGTATAC-MGB-3′	Tandem repeat units M61098	27
	SRA1	5′-CCACGCTCTCGCAAATAATCT-3′		
	SRS2	5′-CAACCGTTCTATGAAAATCGTTGT-3′		
	SRP	5′-FAM-TCCGAAACCACTGGACGGATTTTTATGAT-TAMRA-3′		
Strongyloides stercoralis	Stro-1530F	5′-GAATTCCAAGTAAACGTAAGTCATTAGC-3′	18s	28
	Stro-1630R	5′-TGCCTCTGGATATTGCTCAGTTC-3′		
	Stro-1586T	5′FAM-ACACACCGGCCGTCGCTGC-TAMRA-3′		
Taenia solium	Tsol_ 145F	5′ATGGATCAATCTGGGTGGAGTT-3′	ITS	29
	Tsol_ 230R	5′-ATCGCAGGGTAAGAAAAGAAGGT-3′		
	Tsol_169Tq	5′-FAM-TGGTACTGCTGTGGCGGCGG-TAMRA-3′		
Taenia saginata	Tsag_F529	5′-GCGTCGTCTTTGCGTTACAC-3′	ITS	29
	Tsag_R607	5′-TGACACAACCGCGCTCTG-3′		
	Tsag_581Tq	5′-FAM-CCACAGCACCAGCGACAGCAGCAA-TAMRA-3′		
Trichuris trichiura	TrichF	5′-TTGAAACGACTTGCTCATCAACTT-3′	18s	30
	TrichR	5′-CTGATTCTCCGTTAACCGTTGTC-3′		
	TrichP	5′-FAM-CGATGGTACGCTACGTGCTTACCATGG-TAMRA-3′		
Enterobius vermicularis	EnterF	5′-TTTCCAAGCCACAGACTCAC-3′	5S rRNA region	Designed in this study
	EnterR	5′-ATTGCTCGTTTGCCGATTAT-3′		
	EnterP	5′-FAM-TCATGTCTGAGCCGGAACGAGA-TAMRA-3′		

PCR = polymerase chain reaction.

parallel to validate the negative results. Real-time PCR results were considered negative when the Ct value was more than 38 or no amplification curve was obtained. The limit of the detection was set at cycle 38 because for some parasites, previous studies have reported amplification until cycle 37.[10] For those without information on the limit of detection, late amplification of the 10-fold diluted plasmids showed the threshold around cycle 36–37. All the samples above cycle 35 were retested from the initial eluate to confirm the result.

Data analysis. The data were entered into Excel TM and analyzed with the TM R2.15.0 software (R Foundation for Statistical Computing, Vienna, Austria). Qualitative variables were described in terms of numbers, percentage of data provided, and quantitative data in terms of means with standard deviation. Statistical comparisons were made using the χ^2 test or Fisher's test depending on the conditions of applicability. The test was considered significant if the P value was less than 0.05. Cases were defined as patients experiencing gastrointestinal symptoms including diarrhea (three or more loose or liquid stools per day), abdominal pain, gastroenteritis syndrome (diarrhea + vomiting), pruritus, and dysenteric syndrome. Patients without gastrointestinal symptoms were considered as controls. When comparing the techniques, total agreement statistics (in percent) were calculated as well as the kappa coefficient in the case of discrepancies between the two methods. The kappa agreement level was interpreted as follows: $\kappa < 0.20$ Poor, 0.21–0.40 Fair, 0.41–0.60 Moderate, 0.61–0.80 Good, and 0.81–1.00 Very Good.[31]

Ethics statement. All aspects of this study were approved by the National Ethical Committee of Senegal (agreement no. 00000121-MSAS/DPRS/CNERS). Written informed consent was not obtained from patients in this study because it is not necessary for stool sample collection according to local laws and regulations in Senegal and France. However, oral consent was obtained from patients, including parents on behalf of children and patient medical data were anonymized. Patients were treated according to microscopic results.

RESULTS

Patients, microscopic examination, and real-time PCR results. Overall, 103 stool samples were collected from patients attending health facilities, but 98 were retained in the analysis process. The baseline characteristics of patients enrolled are summarized in Table 2.

The age of patients varied from 1 to 76 years along with a mean age at 26.4 ± 15.2 years. There were 44 (44.9%) patients with gastrointestinal symptoms. The most common clinical signs were diarrhea (16.3%) and abdominal pain (19.3%). Patients with gastrointestinal symptoms were considered as cases and patients without these symptoms as control.

Microscopic examinations yielded 37 (37.7%) positive cases and 61 (62.2%) negative cases. The different coinfections identified by microscopic methods and real-time PCR are summarized in Table 3. In real-time PCR assays, all negative controls run along with samples yielded negative results after testing. Plasmid DNAs used as positive controls allowed us to validate the primers and probes

TABLE 2
Baseline characteristics of enrolled patients from Senegal

	Number	Percentage	95% CI
Age			
Under 5 years	5	5.1	1.6–11.5
5–15 years	10	10.2	5–17.9
16–25 years	35	35.7	26.2–46.03
26–35 years	27	27.5	19.01–37.5
36–45 years	10	10.2	5–17.9
Over 45 years	11	11.2	5.7–19.2
Gender			
Male	51	52.04	41.7–62.2
Female	47	47.9	37.7–58.2
Gastrointestinal symptoms			
Yes	44	44.9	34.8–55.2
No	54	55.1	44.7–65.1
Signs			
Diarrhea	16	16.3	9.6–25.1
Abdominal pain	19	19.3	12.1–28.6
Gastroenteritis	7	7.1	2.9–14.1
Pruritus	1	1.02	0.03–5.5
Dysenteric syndrome	1	1.02	0.03–5.5
Microscopic results			
Positive	37	37.7	28.1–48.1
Negative	61	62.2	51.8–71.8
Real-time PCR results			
Positive	72	73.5	63.5–81.8
Negative	26	26.5	18.1–36.4

CI = confidence interval; PCR = polymerase chain reaction.

for parasites presenting negative results in all clinical samples. All primers/probes were specific to its respective parasite. There was no amplification of target genomic DNA from the other parasites. Overall, 72 (73.5%) clinical samples were positive with real-time PCR including single and multiple infections as shown in Table 3.

TABLE 3
Presentation of coinfections identified by microscopic examination and real-time PCR assays

	Number
Microscopic examination	
Ascaris lumbricoides + *Trichuris trichiura*	2
A. lumbricoides + *Entamoeba histolytica*	1
Real-time PCR assays	
A. lumbricoides + *Strongyloides stercoralis* + *Blastocystis* spp.	1
A. lumbricoides + *Trichuris trichiura*	1
A. lumbricoides + *Trichuris trichiura* + *Blastocystis* spp.	1
A. lumbricoides + *Trichuris trichiura* + *Blastocystis* spp. + *Giardia intestinalis*	1
A. lumbricoides + *Blastocystis* spp.	3
A. lumbricoides + *Enterocytozoon bieneusi*	1
Ancylostoma duodenale + *Schistosoma mansoni*	1
Blastocystis spp. + *Dientamoeba fragilis*	3
Blastocystis spp. + *Taenia saginata*	1
E. histolytica + *Blastocystis* spp.	1
G. intestinalis + *Enterocytozoon bieneusi*	1
G. intestinalis + *Blastocystis* spp.	2
G. intestinalis + *E. histolytica* + *Blastocystis* spp.	1
G. intestinalis + *Necator americanus* + *Blastocystis* spp.	1
G. intestinalis + *T. saginata* + *Blastocystis* spp.	1
Cystoisospora belli + *Blastocystis* spp.	1
Cystoisospora belli + *Cyclospora cayetanensis*	1
S. stercoralis + *Blastocystis* spp.	1
T. saginata + *Blastocystis* spp.	1
Trichuris trichiura + *S. stercoralis*	1

PCR = polymerase chain reaction.

Comparison of real-time PCR with microscopy. The overall positive rate was 73.5% (72/98) in real-time PCR assay versus 37.7% (37/98) in microscopy methods ($P < 0.001$). Both techniques detected more parasites in the "cases" group than in the "control" group ($P < 0.05$), as shown in Figure 1. However, the real-time PCR assay was more sensitive than microscopy for parasite detection in patients without gastrointestinal symptoms ($P < 0.05$) with a sensitivity of 57.4% (31/54) versus 18.5% (10/54), respectively. In terms of polyparasitism, the real-time PCR assay was able to detect more coinfections than microscopy methods (25.5% versus 3.06%), as shown in Figure 1. Most of these coinfections (19/25) consisted of two parasites, with three parasites in five cases and four parasites in one case as shown in Table 3. In the protozoa group, *Blastocystis hominis* and *Giardia intestinalis* were the most commonly detected species by both methods followed by *E. histolytica* (Table 4). *Dientamoeba fragilis* was detected only by real-time PCR and most cases (3/4) were observed in patients without gastrointestinal symptoms. *Cyclospora cayetanensis* was also detected only by real-time PCR. No cases of *Encephalocytozoon intestinalis* were detected by either technique. As shown in Table 4, the real-time PCR assay yielded greater detection rates than microscopy in the identification of protozoan species except for *Cryptosporidium* sp. (similar results).

In the helminths group, the real-time PCR assay was also more sensitive than microscopy in the detection of species as described in Table 4, except for *Schistosoma mansoni* where the two methods gave similar results. Hookworm species (*Ancylostoma duodenale* and *N. americanus*) and *S. stercoralis* were only detected by real-time PCR. There was no case of *T. solium*. In Cestoda, the number of *T. saginata* detected (3/54) was greater in patients without any gastrointestinal symptoms than in the "case" group (1/44).

Performance level of the two methods. By comparing the two methods, the agreement was perfect (100%) between real-time PCR and microscopy in the identification of *Balantidium coli*, *Cryptosporidium* sp., *Encephalitozoon intestinalis*, *Enterobius vermicularis*, *Hymenolepis diminuta*, *Schistosoma mansoni*, and *T. solium*; however, total agreement between the two methods varied from 64.2% to 98.9% for the other species with discrepant results as described in Table 4.

To assess the sensitivity of this molecular method in nonendemic regions, we compared the performance of the real-time PCR assay in detecting intestinal parasites in the microscopy-negative samples from Senegal with microscopy-negative samples collected from Nice and Marseille in France as described in Table 5. The number of positive cases by real-time PCR was greater among microscopy-negative specimens from Senegal (60.6%). However, the assay allowed us to detect parasites in 15 of 46 patients from Marseille (32.6%) and nine of 48 patients from Nice (18.7%). *Blastocystis* spp. was the most frequently species detected in these samples.

DISCUSSION

Many real-time PCR assays for the detection of intestinal parasites have been developed to date, particularly those based on multiplex systems. Among these, the available

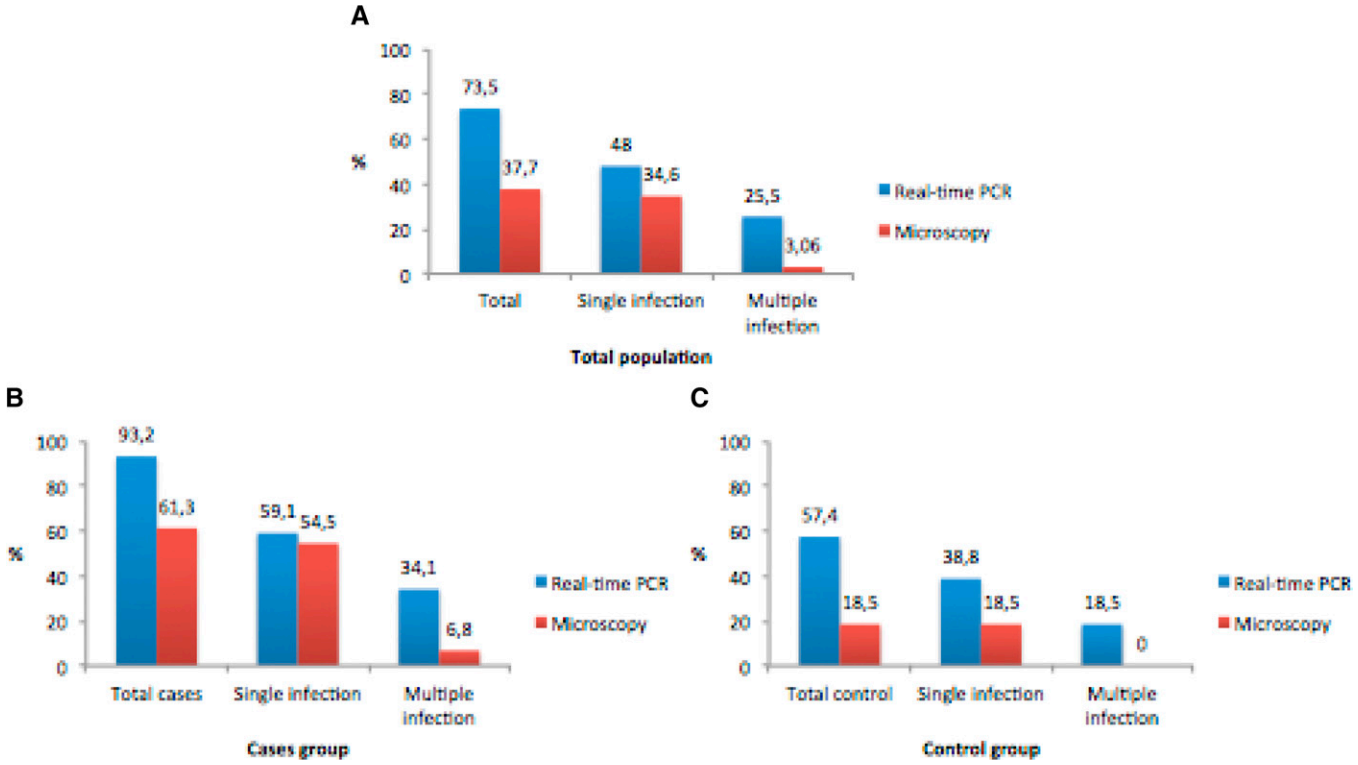

FIGURE 1. Overall comparison of real-time polymerase chain reaction (PCR) vs. microscopy. This figure appears in color at www.ajtmh.org.

commercial kits detect only three or four parasites along with other pathogens.[32–38] In this study, we assessed the performance of a multiparallel real-time PCR assay compared with microscopic methods for the detection of 20 gastrointestinal parasites including protozoa and helminths. The large number of real-time PCR assays tested in this study demonstrated the ability to detect an important number of parasites without technical expertise in parasitology.

The high positivity rate in this study observed with real-time PCR assays compared with microscopy confirms the results of previous studies showing the superiority of real-time PCR in the detection of intestinal parasites.[8–10]

TABLE 4
Comparison of real-time PCR vs. microscopy in the detection of protozoa and helminths in stool samples

	Real-time PCR *n* (%)			Microscopy *n* (%)				
	Total *N* = 98	Cases *N* = 44	Control *N* = 54	Total *N* = 98	Cases *N* = 44	Control *N* = 54	Total agreement* (%)	Kappa
Protozoa								
Balantidium coli	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)		
Blastocystis spp.	47 (47.9)	23 (52.2)	24 (44.4)	12 (12.2)	7 (15.9)	5 (9.2)	63 (64.2)	0.26
Cryptosporidium sp.	1 (1.02)	1 (2.2)	0 (0)	1 (1.02)	1 (2.2)	0 (0)		
Cyclospora cayetanensis	1 (1.02)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	97 (98.9)	0
Dientamoeba fragilis	4 (4.08)	1 (2.2)	3 (5.5)	0 (0)	0 (0)	0 (0)	94 (95.9)	0
Encephalocytozoon intestinalis	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)		
Enterocytozoon bieneusi	2 (2.04)	2 (4.5)	0 (0)	0 (0)	0 (0)	0 (0)	96 (97.9)	0
Entamoeba histolytica	4 (4.08)	2 (4.5)	2 (3.7)	3 (3.06)	2 (4.5)	1 (1.8)	93 (94)	0.14
Giardia intestinalis	12 (12.2)	8 (18.1)	4 (7.4)	7 (7.1)	5 (11.3)	2 (3.7)	91 (92.8)	0.55
Cystoisospora belli	3 (3.06)	2 (4.5)	1 (1.8)	1 (1.02)	1 (2.2)	0 (0)	96 (97.9)	0.40
Helminths								
Ancylostoma duodenale	1 (1.02)	0 (0)	1 (1.8)	0 (0)	0 (0)	0 (0)	97 (98.9)	0
Ascaris lumbricoides	12 (12.2)	11 (25)	1 (1.8)	10 (10.2)	9 (20.4)	1 (1.8)	96 (97.9)	0.85
Enterobius vermicularis	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)		
Hymenolepis diminuta	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)		
Necator americanus	1 (1.02)	1 (2.2)	0 (0)	0 (0)	0 (0)	0 (0)	97 (98.9)	0
Strongyloides stercoralis	3 (3.06)	2 (4.5)	1 (1.8)	0 (0)	0 (0)	0 (0)	95 (96.9)	0
Schistosoma mansoni	1 (1.02)	0 (0)	1 (1.8)	1 (1.02)	0 (0)	1 (1.8)		
Taenia saginata	4 (4.08)	1 (2.2)	3 (5.5)	1 (1.02)	1 (2.2)	0 (0)	95 (96.9)	0.33
Taenia solium	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)		
Trichuris trichiura	8 (8.1)	7 (15.9)	1 (1.8)	4 (4.08)	4 (9.09)	0 (0)	94 (95.9)	0.58

PCR = polymerase chain reaction.
* Total agreement is calculated by the sum of true positive and true negative cases (confirmed by both techniques) divided by the total number of patients.

TABLE 5
Comparison of the performance of real-time PCR for the detection of parasites in microscopy negative samples from Senegal and negative controls from France

No. of real-time PCR positive cases/total microscopy-negative samples (%)	Senegal *N* = 61	France *N* = 94
	37/61 (60.6%)	24/94 (25.5%)
Protozoa		
Balantidium coli	0	0
Blastocystis spp.	24	19
Cryptosporidium sp.	0	1
Cyclospora cayetanensis	1	0
Dientamoeba fragilis	2	1
Encephalocytozoon intestinalis	0	0
Enterocytozoon bieneusi	1	1
Entamoeba histolytica	2	3
Giardia intestinalis	5	0
Cystoisospora belli	2	0
Helminths		
Ancylostoma duodenale	1	0
Ascaris lumbricoides	2	1
Enterobius vermicularis	0	0
Hymenolepis diminuta	0	0
Necator americanus	1	0
Strongyloides stercoralis	3	2
Schistosoma mansoni	0	0
Taenia saginata	3	0
Taenia solium	0	0
Trichuris trichiura	4	1

PCR = polymerase chain reaction.

Moreover, a similar molecular study conducted recently in the United States, using stool samples collected from western Kenya, has demonstrated that some parasites including *Ascaris lumbricoides* and *N. americanus* can be detected by real-time PCR with a high sensitivity rate (98% for both parasites) compared with microscopy (70 and 32%, respectively).[39] Furthermore, the real-time PCR assay applied in our study showed a significantly higher sensitivity rate compared with microscopy in the detection of parasites in asymptomatic patients. This can be explained by the ability of the real-time PCR assay to detect low DNA copy numbers in samples with low egg counts, with as few as 10 copies for some parasite species or 0.013 ng/μL.[39] Another explanation may be the real-time PCR's ability to detect DNA at any lifecycle stage (e.g., larvae), whereas identification by microscopy is optimized for a single stage.[10] This aspect will be useful in the control of these infections, which are considered to be neglected tropical diseases, either by identifying the reservoir or assessing both the level of transmission and the efficiency of deworming programs. Interestingly, Easton and others have showed that the prevalence of many parasites detected by real-time PCR remains higher than that detected by microscopy even after treatment,[39] demonstrating its benefit for low-level parasite detection after antiparasitic therapy. Moreover, the limit of detection and species specificity of detection can be improved by using next-generation sequencing to design assays that target noncoding, high-copy-number repetitive sequences as described by Pilotte and others.[40]

Another finding is the relatively high level of parasitic coinfections detected by real-time PCR compared with microscopy in this study. This finding is interesting as polyparasitism represents an important factor in the process of selecting antiparasitic drugs for mass drug administration.[10,41] Coinfections between helminths and protozoa observed in this study emphasized the need to target some parasites such as *G. intestinalis* in mass drug administration programs, which are currently directed at soil-transmitted helminths.[10]

Among protozoan species observed in this study, *Blastocystis* spp. and *G. intestinalis* were the most common parasites detected by both methods. The first one, *Blastocystis* spp., was detected at a high rate (47.9%) in this study. These high rates of *Blastocystis* have already been reported in rural areas of Senegal where the authors describe a very high prevalence of up to 100%.[42] This high rate has never been reported elsewhere according to the authors. The high prevalence of this parasite in the gut continues to raise real questions about its pathogenicity. For *G. intestinalis*, the results obtained in this study agree with previously reported data describing this parasite as one of the most common protozoan pathogens in Senegal.[43] The remaining protozoans were detected at different percentages according to the method used. Among them, we noted a high rate of *D. fragilis* in asymptomatic patients. The pathogenicity of this parasite has been controversial since its discovery. However, many studies have linked this parasite to the occurrence of gastrointestinal symptoms, especially in children.[9,44] Therefore, introducing molecular methods for detecting this small protozoan will be useful in monitoring it.

In the diagnosis of helminths, both methods were able to easily detect *A. lumbricoides*, *Trichuris trichiura*, and *Schistosoma mansoni* with little difference observed in terms of sensitivity, as diagnosis appears to be simple (except at low concentration) compared with the difficult microscopic detection of protozoan species.[9] However, the real-time PCR assay was only able to detect hookworm species and *S. stercoralis*. This result could be explained by the fact that no specific concentration techniques were used for the identification of *Strongyloides* larvae in this study. Indeed, diagnosis of hookworm and *S. stercoralis* infection is difficult due to the small numbers of ova and larvae available in the feces.[9] Therefore, multiple stools should be tested and sometimes specific concentration techniques, such as the Baermann method, are necessary to increase the sensitivity rate.[45] Unfortunately, these methods are not always used in routine diagnosis and are not available in the field for surveys, leading to the underestimation of infections during epidemiological investigations. Thus, real-time PCR assays seem to be a suitable method for assessing the burden of parasitic infections and the efficacy of ongoing mass deworming programs in the field during epidemiological surveys.

The multiparallel real-time PCR assays tested in this study allowed the detection of 20 gastrointestinal parasites with the same standard operating procedures. However, the time and the cost needed to test 20 parasites per sample can be a limitation in the future. For example, based on the costs of reagents (without DNA extraction and without equipment and labor costs), we estimated that real-time PCR identification (total volume of 20 μL) costs approximately 2€ per parasite and per run (8€ to run four PCRs). So, multiplexing different target DNA offers savings in terms of costs as the reagent costs can be decreased from 8 to 2,18€ by testing four parasites in one PCR run. It can also

offer savings in terms of labor time as reported by Liewellyn and others.[31] Studies to assess the performance of real-time PCR for multiple targets (four to five parasites) in the same run are planned in the near future.

The increased sensitivity of real-time PCR in the detection of parasites in samples from Senegal was also confirmed by the results obtained from Marseille and Nice. The identification of parasites in patients with negative results in microscopy has demonstrated the superiority of real-time PCR compared with microscopy even in regions with a low prevalence of parasitic infections. Most of the parasites detected were protozoa, particularly *Blastocystis* spp. which is described as very difficult to identify by microscopic methods. There was also one case of *Cryptosporidium* sp., one case of *Enterocytozoon bieneusi*, and one case of *D. fragilis* in the control group. It is worthy to note that we did not use herein a permanent stained slide, which is particularly important for detecting certain protozoan parasites, such as *D. fragilis* and *B. hominis,* by microscopic examination. This could explain the superiority of the molecular assays in identifying these parasites in control groups.

Despite the high sensitivity rate observed with real-time PCR assay, the traditional parasitological diagnosis using microscopic tools remains an important method due to its low cost, particularly in endemic low-resource settings, and due to its ability to detect pathogens that are not targeted in the specific real-time PCR assay.

In conclusion, the real-time PCR assay described in this study appears to be a promising tool for diagnosing parasitic infections in laboratories and in the field when evaluating the efficacy of mass drug administration programs currently implemented in many resource-poor settings.

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