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Research Article

Detection of Intestinal Parasites in Stool Samples by Microscopy and Real-Time PCR in Children with Vulnerable Living Conditions in Dakar, Senegal

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ABSTRACT

Background: Intestinal Parasitic Infections (IPIs) are considered a serious public health problem and widely distributed worldwide, mainly in urban and rural environments of tropical and subtropical countries. Globally, soil-transmitted helminths and protozoa are the most common intestinal parasites. Decreasing the prevalence of IPIs is one of the main aims of health services in these countries. This study was designed to determine the current status of IPIs in children with vulnerable living conditions by microscopy and PCR.

Methodology/main findings: A cross-sectional population-based survey was conducted. One stool sample per participant (n=253) was examined by direct smear, Formal-Ether Concentration (FEC), and real-time PCR. It was found that 17.39% harboured at least one helminth while 12.64% harboured two helminths or more. Among the microscopic techniques, FEC was able to detect the broadest spectrum of parasite species. However, FEC also missed a considerable number of infections, notably *S. stercoralis* and *G. intestinalis*. PCR outperformed microscopy in terms of sensitivity and range of parasite species detected.

Conclusion: It was shown that intestinal parasites, especially helminths were omnipresent in our population studies. Classical techniques such as FEC are useful for the detection of some intestinal helminth species, but they lack sensitivity for other parasite species. PCR can detect intestinal parasites more accurately but is generally not feasible in resource-poor settings, at least not in peripheral labs. Hence, there is a need for a more field-friendly, sensitive approach for on-the-spot diagnosis of parasitic infections.

Keywords: Helminths; Protozoa; RT-PCR; Vulnerable children

INTRODUCTION

Intestinal Parasitic Infections (IPIs) remain a public health problem in many communities, especially among children living in developing countries. It is estimated that more than 2 billion people worldwide are infected with IPI and more than half of the world's population is at risk of infection [1,2]. In sub-Saharan countries, 866 million people are infected with Soil-Transmitted Helminths (STH), the majority of these infections occurring in schoolchildren. The World Health Organization (WHO) estimates that 450 million people are sick [3,4]. In addition to morbidity and mortality, infections with intestinal parasites have been associated with growth retardation, underweight, physical weakness and poor school performance in schoolchildren [5,6]. As part of a long-term goal to eliminate intestinal parasitic infections as a public health problem by 2020, WHO has recommended Mass Drug Administration (MDA) with a single oral dose of mebendazole or albendazole given periodically to children preschool and school age [7,8]. This strategy is currently being implemented in many countries including Senegal. Microscopic examination of stool samples is the most widely used diagnostic approach for the detection of intestinal parasites. First of all, a direct microscopic examination is carried out by mixing a small amount of faeces with physiological sodium chloride solution (0.9%). Next, various stool concentration techniques based on the use of sedimentation or flotation with formalin-ether concentration technique are performed to increase sensitivity [9,10]. In addition, an accurate microscopic diagnosis depends on the experience of the laboratory microscopist and the concentration of parasitic material in the sample. Finally, certain species of parasites such as *Entamoeba histolytica*, *Cryptosporidium sp* and *Strongyloides stercoralis*, which are responsible for serious infections, are often misdiagnosed even when concentration techniques are used [11,12]. Although microscopic examination of stool specimens remains the gold standard for the diagnosis of parasitic infections, it is lengthy, laborious and requires substantial technical expertise. In contrast, rapid diagnostic methods such as Polymerase Chain Reaction (PCR)-based assays were developed to improve the sensitivity and specificity

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of detection of enteric parasites, including helminths, protozoa and microsporidia [13,14]. To overcome these shortcomings, molecular techniques were suggested as a complementary process and may be an alternative to microscopic examination. Indeed, the conventional real-time Polymerase Chain Reaction (PCR) has turned out to be sensitive and precise for the detection of helminths and intestinal protozoa [14]. These techniques have the advantage of detecting low levels of parasites, improving the identification of infected persons and evaluating treatment effects by quantification [15]. This Work is the first prospective, Senegal epidemiological study aiming at estimating the occurrence of three helminth *Ascaris*, *Strongyloides* and *Trichocephale* and one protozoan *Giardia intestinalis*. Microscopy and qPCR results were also compared.

MATERIALS AND METHODS

This study was carried out in Koranic schools in the Dakar region between January and August 2018.

Study sites

A mapping of this area was prepared and the Koranic schools were randomly selected. In order to obtain a random and evenly geographically distributed, Koranic schools and a logistically and technically feasible laboratory sample size were selected. In this way, all participants, i.e. a total of 15 Koranic schools were approached to participate. In the field, these schools were identified thanks to the health district relays. Schoolchildren who did not provide enough faeces for all procedures were excluded from the study. Collection was done on 253 of the schoolchildren who gave informed consent and who provided enough faeces to be included in the study.

Microscopy

Stool samples were collected from all "Darras" (Koranic schools) members between 8 a.m. and 2 p.m. Arrived at the laboratory of the Faculty of Medicine the stools were immediately examined. Two well-trained microscopists followed the laboratory procedures and on average no more than 15 stool samples were processed per day with a double reading of the slide to ensure high quality microscopic results. Several approaches were used for the detection of cysts and oocysts of protozoa and of helminth eggs and larvae. Microscopic techniques included direct smear and the modified Ritchie Method. For the direct smear, ~2 mg of faeces was mixed with normal saline on a microscopy slide and examined for helminth eggs. Another ~2 mg of faeces was mixed with a drop of iodine and examined for protozoan cysts. For the modified Ritchie Method, one gram of fecal matter was thoroughly mixed with 8 ml of 10% formalin. An FPC filter with a 15 ml tube was attached to the tube containing this mixture. After filtering the suspension in the empty tube, 3 ml of ether was added to the filtrate. This mixture was then shaken vigorously for 1 minute and centrifuged at 500 × g for 2 minutes. A thick, unstained sediment frame was used for the detection of helminth eggs and larvae. For protozoan cysts, a thin moist layer of iodine-stained sediment was used.

Real time PCR assay

At the laboratory of the department of Parasitology (Faculty of Medicine, Cheikh Anta Diop University), an aliquot (~1 g) of each stool sample was sieved and mixed with 3 volumes of 96% ethanol for storage for performing Real-time PCR. Then the samples were stored at -20°C until the detection and quantification of parasitic DNA loads by real-time PCR. Isolation, amplification and detection of DNA were performed blind from previous microscopic results. DNA was extracted from the stool samples using a modified method of the Omega Bio-tek Extraction Kit [16,17]. For DNA isolation, 200 mg of faeces was collected on Eppendorf tubes of 1.5 ml and 30 µl of proteinase K and 250 µl of BL Buffer are added, then vortex for 15 seconds and incubate at 65°C for 10 minutes the pellet was washed with a solution with 260 µl of absolute ethanol, and vortex at maximum speed. The entire sample was transferred to the column, 500 µl of HBC Buffer were added followed by successive washings with 700 µl DNA washing buffer then a 100 µl Elution Buffer heated to 65°C. The DNA collected is stored at -20°C.

A total of 5 PCR targets were included and 5 µl of DNA was used in each real-time PCR. Real-time PCR reactions were performed using total volumes of 20 µl 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 (Table 1). The analyses were carried out using a CFX96™ Real-Time PCR detection test (Bio-Rad Life Science, Marnes-la-Coquette, France). Amplification reactions were performed as follows: 95°C for 15 minutes followed by 44 cycles of 60°C for 0.5 minutes and 72°C for 1 minute. Negative and positive control samples were included in each PCR run. The PCR output from this system consisted of a Cycle Threshold value (Ct), representing the amplification cycle in which the level of the fluorescent signal exceeded the background fluorescence. Therefore, low Ct values correspond to high parasite-specific DNA loads in the test sample, and vice versa. The maximum Ct value was set at 37.

Table 1: List of primers and probes (Applied Biosystems UK) used in this study for the detection of 5 intestinal parasites by real-time PCR.

Parasites	Name	Primes/Probes	Target region	Reference
Protozoa				
Giardia lamblia (intestinalis or duodenalis)	*Giardia*-80F	5'-GACGGCTCAGGACAACGGTT-3'	18s	[18]
	Giardia-127R	5'-TTGCCAGCGGTGTCCG-3'		
	Giardia-105T	5'-FAM-CCCGCGGCGGTCCCTGCTAG-TAMRA-3'		
Helminths				
Ascaris lumbricoides	Alum96F	5'-GTAATAGCAGTCGGCGGTTTCTT-3'	ITS-1	[19]
	Alum183R	5'-GCCCAACATGCCACCTATTC-3'		
	Alum124T	5'-FAM-TTGGCGGACAATTGCATGCGAT-TAMRA-3'		
Strongyloides stercoralis	Stro-1530F	5'-GAATTCCAAGTAAACGTAAGTCATTAGC-3'	18s	[20]
	Stro-1630R	5'-TGCCTCTGGATATTGCTCAGTTC-3'		
	Stro-1586T	5'FAM-ACACACCGGCCGTCGCTGC-TAMRA-3'		
Trichuris trichiura	TrichF	5'-TTGAAACGACTTGCTCATCAACTT-3'	18s	[21]
	TrichR	5'-CTGATTCTCCGTTAACCGTTGTC-3'		
	TrichP	5'-FAM-CGATGGTACGCTACGTGCTTACCATGG-TAMRA-3'		

Statistical analysis

Data were entered on Excel and analysis performed using Stata 13. Variables were compared using Fisher Exact test Chi square depending on the conditions of application of these tests were estimated to assess the performance of microscopy and RT-PCR for the detection of parasitic infections. Agreement of the two methods was assessed using Cohen's Kappa test.

Ethics statement

This study was conducted according to the declaration of Helsinki and existing national legal and regulatory requirements. The protocol was reviewed and approved by the Senegalese Ethics Committee at the Cheikh Anta Diop University. Approval number 0258/2017/CER/UCAD. Informed consent of parent or legal representative was required prior to the participation in the study. To respect confidentiality, an identification code was given to each participant.

RESULTS

Socio demographic characteristics of the study population

In total, 253 people participated in this study, 221 (87.3%) boys and 32 (12.65%) girls (age group=4 to 21 years, mean age=11.43 years). Participants were stratified by age and sex. There were 105 (41.5%) participants aged 1 years to 10 years, 148 (58.5%) aged 11 years to 21 years. A total of 44 people (17.39%) had a positive result by microscopy and 94 or 37.15% had a positive result by RT-PCR (Table 2).

Table 2: Baseline characteristics of the study population.

	Number	Percentage	95% IC
Age			
≤ 10	105	41.50%	[35.3-47.8]
>10	148	58.50%	[52.1-64.6]
Gender			
Male	221	87.35%	[82.6-91.1]
Female	32	12.65%	[8.8-17.3]
Microscopic result			
Positive	44	17.39%	[12.9-22.6]
Negative	209	82.38%	[80.4-89.4]
Real-time PCR result			
Positive	94	37. 15 %	[31.3-43.5]
Negative	159	62.45%	[56.4-68]

Direct comparisons between diagnostic techniques on individual samples from all participants were made using p-value and Kappa agreement statistics where applicable (Table 3). The results show a good, moderate and fair match for *Ascaris*. For *Giardia* and *Trichuris*, respectively, the difference in detection was statistically significant (p=0.0009, p=0.003). For all parasites analysed, the RT-PCR identified a large number of positive samples not detected by microscopy (25 *Ascaris*, 11 *Strongyloides*, 47 *Giardia* and 12 *Trichirus*), while a small number of positive samples by microscopy were not identified as positive by RT-PCR (7 *Ascaris*, 5 *Giardia* and 3 *Trichirus*).

Table 3: RT-PCR and microscopy parasite prevalence agreement statistics.

	PCR	Microscopy		Kappa	p
		Positive	Negative		
Ascaris	Positive	23	25	0.51	0
	Negative	7	198		
Strongyloide	Positive	0	11	0	-
	Negative	0	242		
Trichuris	Positive	2	12	0.18	0.003
	Negative	3	236		
Giardia	Positive	6	41	0.14	0.0009
	Negative	5	200		

Microscopy detected an association of *Ascaris* and *Trichocephalosis* infection. Most of the coinfections were detected by RT-PCR which was able to detect both double and triple parasitisms, the association between helminth (*Ascaris* and *Trichocephalosis*) but also between helminth and protozoan (*Strongyloidosis*+*Giardia*). The association between helminth and protozoan could be detected at 9.09% (Table 4).

Table 4: Presentation of coinfections identified by microscopic examination and real-time PCR assays.

	Number
Microscopic examination	
Ascaris¬+Trichocephalose	1
Real Time PCR	
Ascaris+Trichocephalose	5
Ascaris+Strongyloide	6
Ascaris+*Giardia*	12
Strongyloide+Trichoceohalose	1
Trichocephalose+*Giardia*	2
Strongyloide+*Giardia*	7
Ascaris Strongyloide *Giardia*	4
Ascaris Strongyloide Trichocephalose *Giardia*	1
Ascaris Strongyloide *Giardia*	1

Comparison of the direct examination and the ritchie concentration

Diagnostic sensitivity was estimated for the direct examination and the Ritchie concentration method and for each of the parasite species (Figure 1). The direct examination and the Ritchie method have some sensitivity in detecting every helminth, with the exception of *Strongyloides stercoralis*. The direct smear was inferior to the Ritchie concentration method for the detection of *Trichirus trichiura* and *Ascaris* and *Giardia intestinalis*. Likewise, the direct smear was inferior to the Ritchie method for the detection of *G. intestinalis*.

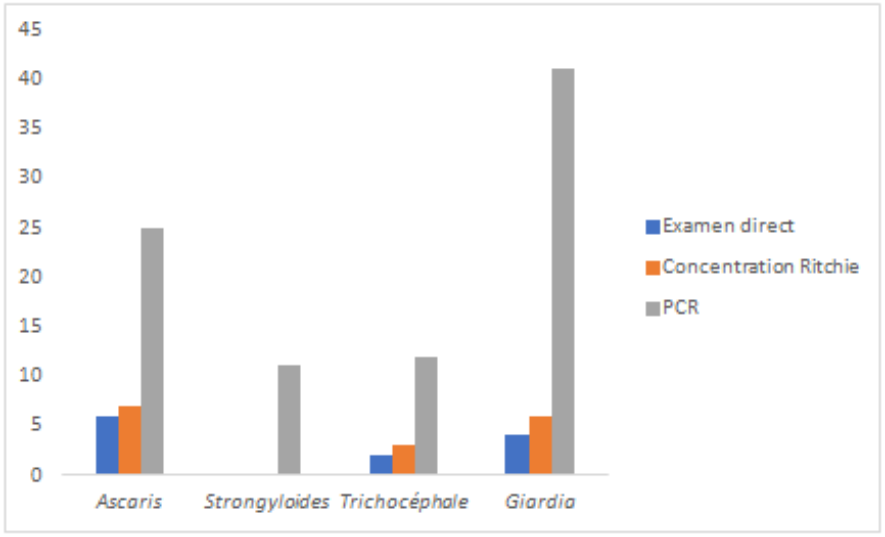

Figure 1: Prevalence of intestinal parasitic infection in the study population based on different diagnostic methods.

Microscopy versus PCR

The sensitivity of PCR for the detection of each of the parasite species tested was greater than that of any of the microscopic techniques applied (Figure 1). This difference was statistically significant p(0.003) for both microscopic methods used for *S. stercoralis*, and *G. intestinalis* and for the direct smear for the detection of *A. lumbricoides*. For some species, the sensitivity of the microscopic technique was much lower than that of PCR. For *G. intestinalis*, the sensitivity of FEC was 4.45% while that of PCR was 18.83%.

DISCUSSION

This study was initiated to get an idea on the distribution of the most common intestinal parasitoses in children with unfavourable living conditions after 4 courses of MDA in the study area [17-20]. However, the diagnostic methods used in prevalence surveys rely on microscopy techniques. The first objective of this study was to obtain an overview of certain intestinal helminths and protozoan infections (*Giardia intestinalis*). The levels of intestinal parasitic infections observed in our study from microscopy are close to the results of other previous studies carried out in rural areas (Keur Socé) by Tine and al which showed 26.2% prevalence for intestinal parasites with a predominance of protozoan parasites [21]. Compared to the urban area in Senegal, similar results showed a prevalence of 26.8% while Ndiaye reported a prevalence of 20.3% [22,23].Similar results are observed in some African countries, namely Kenya (25.6%) and Ethiopia (26.2%) [24,25]. The high positivity rate in our study observed with real-time PCR tests compared to microscopy confirms the results of previous studies showing the superiority of real-time PCR in the detection of intestinal parasites [26,27]. In addition, a similar molecular study recently conducted in the United States, using stool samples from western Kenya, demonstrated that certain parasites (*Ascaris lumbricoides* and *N. americanus*) can be detected by real-time PCR with a high rate of sensitivity (98% for both parasites) compared to microscopy (70% and 32%, respectively) [28].

Our results showed a relatively high rate of coinfections detected by real-time PCR compared to microscopy. This finding is interesting because polyparasitism is an important factor in the selection process of antiparasitic drugs for mass drug administration.

In the diagnosis of helminths, both methods were able to easily detect *A. lumbricoides* and *Trichuris trichiura* with little difference observed in terms of sensitivity, as the diagnosis appears simple (except at low concentration) compared to difficult microscopic detection of species of protozoa [29]. However, the real-time PCR test could only detect *S. stercoralis* species. This result could be explained by the fact that no specific concentration technique was used for the identification of *Strongyloides* larvae in our study. Indeed, the diagnosis of *S. stercoralis* infection is difficult due to the small number of ova and larvae available in the faeces [29]. Therefore, several stool examinations must be performed and sometimes specific concentration techniques, such as the Baermann method, are necessary to increase the rate of sensitivity. Unfortunately, these methods are not always used in routine diagnosis and are not available in surveys, leading to underestimation of infections during epidemiological surveys. Thus, real-time PCR testing appears to be an appropriate method to assess the burden of parasitic infections and the effectiveness of ongoing mass drug distribution programmes in the field during epidemiological surveys.

It is widely recognized that PCR could be particularly useful for the detection of Intestinal parasites in areas of low transmission and in the post-control settings. The co-infection between helminths and protozoa observed in this study highlighted the need to target certain parasites such as *G. intestinalis* in mass drug administration programmes, which are currently against soil-transmitted helminths.

CONCLUSION

The results showed that intestinal parasites, especially helminths, are still present despite MDA in children. However, it is difficult to achieve high diagnostic sensitivity for all species. Conventional techniques such as the Ritchie Modified Concentration Technique are useful for the detection of certain species of helminths, but they lack sensitivity for certain species of parasites. RT-PCR can detect intestinal parasites and polyparasitism more accurately, but is generally not feasible in countries with limited resources, at least not in peripheral laboratories. The real advantage of RT-PCR lies

in its ability to more accurately determine the intensity of infection and the potential to report the results in more understandable "quantitative" terms, which will prove inherently more useful in determining the infection. Success of therapeutic and intervention trials. Therefore, it is necessary to adopt a more sensitive approach in the field for the diagnosis of parasitic infections in order to hopefully interrupt the chain of transmission of intestinal parasitosis.

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