

Strongyloidiasis in humans: diagnostic efficacy of four conventional methods and real-time polymerase chain reaction

Laura Francisca Campo-Polanco^[1], José Mauricio Hernández Sarmiento^[2], Miguel Antonio Mesa^[3], Carlos Jaime Velásquez Franco^[3], Lucelly López^[2], Luz Elena Botero^[1] and Lina Andrea Gutiérrez Builes^[1]

[1]. Grupo Biología de Sistemas, Escuela de Ciencias de la Salud, Facultad de Medicina, Universidad Pontificia Bolivariana, Medellín, Colombia.

[2]. Grupo Salud Pública, Escuela de Ciencias de la salud, Facultad de Medicina, Universidad Pontificia Bolivariana, Medellín, Colombia.

[3]. Sección Reumatología, Clínica Universitaria Bolivariana, Universidad Pontificia Bolivariana, Medellín, Colombia.

Abstract

Introduction: *Strongyloides stercoralis* is an intestinal parasitic nematode that causes hyperinfection and/or a dissemination syndrome in hosts, which is often difficult to diagnose. This study aims to compare the diagnostic efficacy of four conventional methods used to diagnose strongyloidiasis with real-time polymerase chain reaction (qPCR) to detect *S. stercoralis* in fecal samples. **Methods:** We analyzed 143 fecal samples collected from Colombian regions with varying degrees of risk for intestinal infections caused by *S. stercoralis* to assess the validity, performance, overall efficiency, and concordance of the qPCR using a direct stool test, modified Ritchie concentration technique, agar plate culture, and Harada-Mori technique as reference tests. **Results:** While four fecal samples were positive for *S. stercoralis* using conventional methods, 32 were positive via qPCR. The diagnostic sensitivity of the qPCR was 75% [95% confidence interval (CI): 20.07-100%], whereas its specificity, negative predictive value, negative likelihood ratio, and Youden's J index were 78.42% (95% CI: 71.22-85.62%), 99.09% (95% CI: 96.86-100%), 0.32 (95% CI: 0.06-1.74), and 0.53, respectively. In addition, the estimated kappa index between the qPCR and the conventional methods was 0.12 (95% CI: -0.020-0.26). **Conclusions:** The diagnostic sensitivity of qPCR to detect strongyloidiasis is analogous to that of conventional parasitology methods, with an additional advantage of being capable of identifying the parasite DNA at low sample concentrations.

Keywords: *Strongyloides stercoralis*. Intestinal diseases. Helminthology. Molecular diagnostic techniques.

INTRODUCTION

Strongyloides stercoralis is an intestinal nematode that is commonly detected in the tropical and subtropical parts of the world, with a prevalence of 10-40% in tropical countries^{1,2}. Alternating stages of parasitic life, autoinfection cycles, and free life account for the high complexity of the biological cycle of this nematode^{3,4}, which is further exacerbated by high humidity and temperatures of 20-37°C required for its proliferation^{5,6}. In some cases, socioeconomic and environmental factors often converge with high-risk factors, such as extreme poverty, inadequate ecological sanitation, poor excreta disposal, and soil organic debris^{5,7}, which are all associated with a higher prevalence of intestinal infections caused by *S. stercoralis*. In addition, in people

with an impaired cellular immune response, the occurrence of autoinfection cycles of strongyloidiasis might cause hyperinfection and/or the dissemination of larval stages toward other organs, increasing the risk of potentially fatal complications^{6,8,9}.

Typically, strongyloidiasis is diagnosed by identifying the larval stages using methods such as the direct stool test, which is the routinely performed test in clinical laboratories because of its simplicity and fast sample-processing rate. However, the direct stool test exhibits low performance, efficiency, and diagnostic certainty for detecting *S. stercoralis*¹⁰, frequently leading to false-negatives¹¹. Other available methods for detecting *S. stercoralis* are the modified Ritchie concentration technique, isolation or culture on an agar plate, and the Harada-Mori technique and Baermann methods for larval separation^{5,12,13}. Although the diagnostic sensitivities of these methods are higher, these procedures are laborious and time-consuming^{7,14}.

Lately, the polymerase chain reaction (PCR) has been proposed as a valid, reliable, and rapid alternative for the detection of *S. stercoralis*¹⁵⁻²¹. Reportedly, PCR confers multiple

Corresponding author: Dr^a Laura Francisca Campo Polanco

e-mail: laura.campolanco@gmail.com

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advantages, including the ability to precisely detect the genetic material of *S. stercoralis*, even if its deoxyribonucleic acid (DNA) is a free molecule in the analyzed sample, and that its identification does not depend on the viability of the parasite²².

This study aims to compare the diagnostic efficacy of four conventional methods used to diagnose strongyloidiasis with real-time PCR (qPCR) to detect *S. stercoralis* in fecal samples collected from Colombian regions with varying degrees of risk for intestinal infections caused by *S. stercoralis*.

METHODS

Study population

In this study, we collected fecal samples from 143 men and women, using non-probabilistic or convenience sampling, in groups where the Universidad Pontificia Bolivariana School of Health Sciences performed community work. Adult habitants of the rural areas of Chocó and the peri-urban areas of the Chocó and Antioquia departments for >18 year and who consented to participate in the study were included. They were divided into three groups based on geographical zones. The first group comprised of indigenous people from Emberá Dobida and Emberá Chamí ethnic communities living in rural areas of the Department

of Chocó as well as inhabitants of two villages in the municipality of Carmen de Atrato (Chocó) where a health services network was not available (**Figure 1**). The second group comprised of residents of outlying neighborhoods in Quibdó and Medellín (Colombia), where some essential sanitation services, such as pipe water and excreta disposal, were available (**Figure 1**). Finally, the third group comprised of patients with rheumatic diseases who were undergoing immunosuppressive treatment at Clínica Bolivariana (Medellín, Colombia). Individuals who received antiparasitic treatment 3 months before the study period were excluded.

Ethical considerations

All procedures in this diagnostic test evaluation study and data collection methods were in accordance with the fundamental ethical principles regulating the ethical conduct and governed by the Nuremberg Code (1947), the Helsinki Declaration (enacted in 1964, amended in Korea in 2008 and at the 64th ICH's General Assembly held in Fortaleza, Brazil, in 2013), and the national standard 008430 for health research. In addition, the study protocol was approved by the Universidad Pontificia Bolivariana's Health Research Committee (October 21, 2013). We obtained written informed consent from all participants who provided samples in this study.

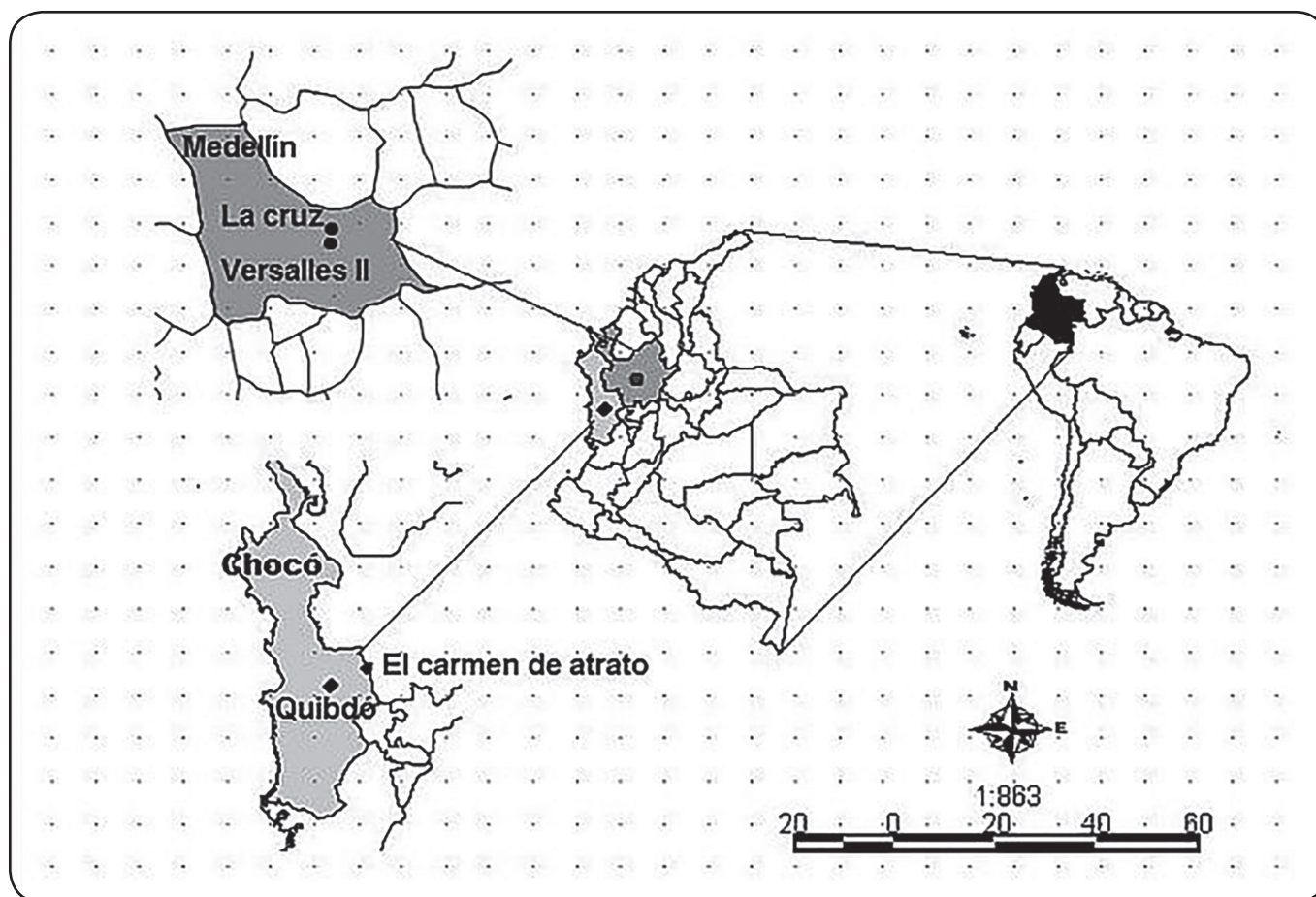


FIGURE 1: A map of the sampled areas in Antioquia and Chocó, Colombia (represented using the DIVA-GIS® software, <http://www.diva-gis.org/>). **DIVA-GIS:** Graphic Information System.

Sample collection and processing using conventional parasitology methods

We collected spontaneous emission fecal samples and divided those into three aliquots as follows: **a)** first aliquot with no added preservatives for setting up tests requiring viability to generate a positive result (e.g., agar plate culture and Harada-Mori technique); **b)** second aliquot with 10% formalin added for preservation, for tests requiring no viability (e.g., direct stool test and modified Ritchie concentration technique); and **c)** third aliquot with storage at -20°C and use in the qPCR. All stool samples were assessed using four conventional parasitology methods – direct stool test, modified Ritchie concentration technique, agar plate culture, and Harada-Mori technique – recommended by the World Health Organization because a unique diagnostic method that could be used as a reference test for the detection of *S. stercoralis* was unavailable²³. Before carrying out the study, the magnitude of the interobserver variability was estimated through a concordance study that facilitated the evaluation of the correlation power between the observers involved in the test readout. This translated into reliability and validity of the results obtained from the readouts of different parasitology methods and into the minimization of measurement errors originating in observer variability. In the end, kappa index values higher than 0.70 were obtained for most of the identified intestinal parasites, which suggest a good correlation among the analysts²⁴⁻²⁶.

Molecular analysis and qPCR based on 18S rRNA gene sequences

A formerly standardized qPCR technique was used for the molecular analysis of the fecal samples tested in this study. Primers reported by Verweij JJ et al.¹⁷, and a TaqMan probe (Custom TaqMan®, MGB (minor groove binder) Probes, Applied Biosystems®, Thermo Fisher Scientific; Massachusetts, United States), marked with FAM™ fluorochrome, as previously described¹⁶, were employed in the amplification of the 18S ribosomal ribonucleic acid (rRNA) gene of *S. stercoralis*. In all tests, an exogenous DNA or IPC Internal Positive control (TaqMan® Exogenous Internal Positive control, Applied Biosystems®, Thermo Fisher Scientific; Massachusetts, United States) was introduced as an internal positive control of the reaction to evaluate the presence of inhibitors in the fecal samples, and ultrapure water was used as a negative control. The qPCR conditions were established by evaluating the sensitivity and analytical specificity of serial dilutions of samples containing *S. stercoralis* larvae as well as the occurrence of cross-reactions with other parasites and amplification inhibitors. The threshold cycle (Ct) value obtained with the standardized qPCR was less than or equal to 29.99 in the positive samples. A Ct value between 30.00 and 34.99 was considered indeterminate, assuming that only samples with a very low parasitic load would show those values²⁷.

We randomly selected a PCR product from one of the positive samples and one from the positive controls to validate the *S. stercoralis* rRNA 18S gene-specific sequence¹⁹. Consequently, we randomly selected seven positive samples

(Ct of 23-29.99), three with Ct values between 30-34.99, and three positive controls. Subsequently, bidirectional sequencing of the PCR product was performed in Macrogen (Maryland, USA). We edited and aligned the obtained sequences using the Geneious® software ver 7.1.7²⁸. Further, we verified the identity using the Basic Local Alignment Search Tool (BLASTn) for the analysis of the consensus sequence in GenBank (blast.ncbi.nlm.nih.gov) and conducted the maximum likelihood molecular phylogeny analysis using the MEGA (Molecular Evolutionary Genetics Analysis) software ver. 6. In addition, we used the Bayesian information criterion²⁹ and Akaike information criterion to select a molecular evolution model. The dendrogram estimation was performed by a heuristic search and a bootstrap resampling with 1,000 pseudo values. Furthermore, we used the same molecular evolution model previously selected for partial sequences of the ribosomal RNA 18S region with the MrBayes software ver. 3.2.2, with the plug-in available in Geneious® ver. 7.1.7²⁸.

Statistical analysis

We conducted a survey to identify epidemiological variables and hygiene-sanitary conditions, and further analyzed the obtained data with Statistical Package for the Social Sciences (SPSS) ver. 24³⁰, both to elucidate the epidemiology and parasitology data by a descriptive analysis of quantitative variables and frequency analysis. Epidemiological data were presented as absolute numbers and percentages, and parasitological data were presented as absolute numbers. In addition, we calculated the sensitivity, specificity, positive and negative predictive values, likelihood ratio, and the kappa index, while comparing the results obtained with the conventional parasitological methods to those with the qPCR. A kappa index closer to 1, calculated using the Epidat 4.1 software³¹, indicated an almost perfect match between the analyzed tests²⁴, and these results were presented in percentages. Furthermore, we performed an adjustment to estimate these same parameters considering the prevalence of this parasitic infection, as reported by Colombia's Ministry of Health (1.5%, according to the national survey of intestinal parasitism)³², and using the Bayes theorem in the Bayesian analysis module of Epidat 4.1.

RESULTS

Characterization of the study population

Among the 143 fecal samples, we collected 72 from the rural zone of Chocó, 57 from the outlying areas of Quibdó and Medellín Cities, and 14 from individuals receiving immunosuppressive treatment. **Table 1** summarizes the hygiene and sanitary, or housing conditions of individuals in rural and urban zones. All participants undergoing immunosuppressive therapy had drinking water, sewerage, pets, and did not walk barefoot.

Conventional parasitology methods

The modified Ritchie concentration technique detected 4/4 positive samples for *S. stercoralis* (three were from the rural zone of Chocó and one from the La Cruz neighborhood in Medellín). The remaining specific traditional methods for the

TABLE 1: Epidemiological characteristics of the study population.

Variables	Rural zone	Urban zone	Overall (%)
Demographics & socio-economic			
Age (years)			
18–29	43	23	51.0
30–44	23	9	25.0
>45	5	20	19.0
no data	3	3	4.7
Sex			
man	32	14	35.6
woman	40	42	63.5
no data	0	1	0.8
Education level			
elementary	18	32	38.7
high school	7	17	18.6
technical	0	1	0.8
college	0	1	0.8
none	47	6	41.0
Hygiene-sanitary conditions			
Potable water for consumption			
yes	1	20	16.3
no	70	35	81.4
no data	1	2	2.3
Type of lavatory			
latrine	15	1	12.4
toilet	12	47	45.7
other	4	7	8.5
none	40	1	31.8
no data	1	1	1.6
Water source			
aqueduct	0	19	14.7
spring	45	31	58.9
water tank	24	6	23.2
other	2	0	1.6
no data	1	1	1.6
Type of residence flooring			
soil	25	10	27.1
cement	1	26	20.9
tile	0	1	0.7
wood	46	19	50.4
no data	0	1	0.7
Personal history			
Have frequent diarrhea?			
yes	45	27	55.8
no	26	28	41.9
no data	1	2	2.3
Water for consumption is boiled?			
yes	40	21	47.2
no	31	35	51.2
no data	1	1	1.6
Animals living in the residence?			
yes	46	40	66.7
no	25	16	31.8
no data	1	1	1.6
Used to walk barefoot?			
yes	62	22	65.1
no	8	34	32.6
no data	2	1	2.3

diagnosis of strongyloidiasis were negative for all samples, except for the isolation on agar plates, in which one of the four positive samples by the modified Ritchie concentration method could be detected with a sensitivity and specificity of 25% (95% CI: 4.6-69.9%) and 100% (95 CI: 97%-100%), respectively. **Table 2** summarizes the parasitological results based on groups and detection methods.

Molecular analysis and qPCR based on 18S rRNA gene sequences

Among the 143 samples, 32 were positive (Ct value < 29.99) according to the qPCR. Of these 32 positive samples, three corroborated the parasitology diagnosis by conventional methods (one sample was from the outlying area of Medellín and the other two were from the rural zone of Chocó). Among the remaining 29 positive samples, two were from the rural zone of Chocó, 24 from the La Cruz neighborhood in Medellín, and three from patients undergoing immunosuppressive treatment. In addition, we classified 16 samples from Chocó, 6 from

the outlying area of Medellín, and 8 from patients receiving immunosuppressive therapy as undetermined (Ct value = 30-34), and 81 samples displayed negative results (**Table 2**).

The analysis of the sequences obtained for the rRNA 18S rRNA 101-bp fragment found in sample I9 and the SF3-positive control amplified by the qPCR revealed an identity percentage of 95% and 96%, respectively, compared with the partial sequence of the same marker reported for *S. stercoralis* in GenBank (access code: M89229; analyzed size: 50bp). Meanwhile, the BLASTn analysis of the sequences obtained for the 18S rRNA 244-bp fragment revealed a percentage of identity with the partial sequence of this same region reported for *S. stercoralis* in GenBank (access code: KM387397) between 99% and 100%. Regarding the phylogenetic analyses based on this molecular marker (analyzed size: 101bp), both dendrograms obtained using the maximum likelihood analysis (**Figure 2**) as well as the Bayesian inference (**Figure 3**) revealed all sequences derived in this study under a single group, forming a polytomy along with all the sequences from the *Strongyloides* genus reported in GenBank.

TABLE 2: The number of positive samples for each parasite, stratified according to the group of subjects analyzed and the diagnostic method used.

Parasite	Diagnostic method														
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	rural zone					outlying zone					Immunosuppressive treatment				
Nematodes															
<i>Strongyloides stercoralis</i>	0	3	0	0	6	0	1	1	0	23	0	0	0	0	3

1: direct stool test; 2: modified Ritchie concentration technique; 3: agar plate culture; 4: Harada-Mori technique; 5: qPCR. **qPCR**: real-time polymerase chain reaction.

TABLE 3: Estimates for the qPCR operative characteristics.

Parameter	Values obtained from test comparison (%)	95% CI	Values adjusted using Bayes's Theorem
Sensitivity	75.0	20.07–100.0	75.0
Specificity	79.1	72.02–86.25	78.4
Positive predictive value	9.4	0.0–21.04	5.2
Negative predictive value	99.1	96.89–100.0	99.5
Percentage of false positives	20.6	14.5–28.5	–
Percentage of false negatives	25.0	4.6–69.9	–
Accuracy	79.2	71.5–85.3	–
Diagnostic odds ratio	11.5	1.15–115.55	–
Youden's index	0.5	–	–
LR(+)	3.6	1.87–6.90	–
LR(-)	0.3	0.06–1.73	–
Pre-test likelihood (prevalence)	2.8	–	–
Validity index	79.0	72.0–86.04	–

qPCR: real-time polymerase chain reaction; **95% CI**: 95% confidence interval; **LR+**: positive likelihood ratio; **LR-**: negative likelihood ratio; **LR**: likelihood ratio.

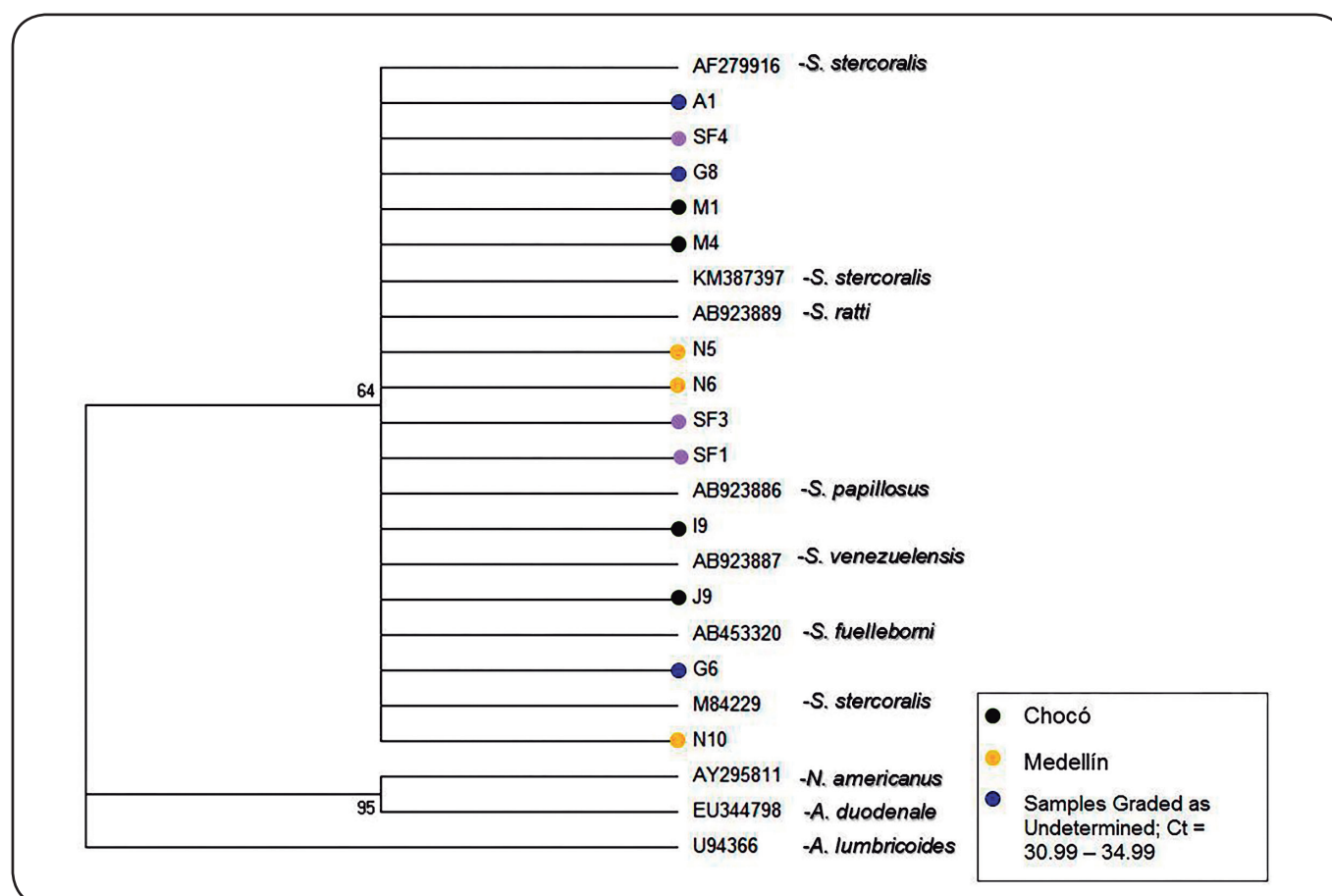


FIGURE 2: Dendrogram obtained by consensus partial sequences of the ribosomal RNA 18S gene from this study reported at the National Center for Biotechnology Information using the maximum likelihood analysis based on the Jukes Cantor model. The value on the branches indicates the bootstrap percentage (1,000 iterations). **Ct:** Cycle threshold; **RNA:** ribonucleic acid.

Diagnostic evaluation of the qPCR

In this study, the qPCR standardized and tested for the diagnosis of *S. stercoralis* exhibited 75% sensitivity (20.07-100%), 78.42% specificity (71.22-85.62%), 9.09% positive predictive value (0.0-20.41%), and 99.09% negative predictive value (99.86-100%). Regarding the matches between the results obtained with conventional parasitological methods, such as direct stool test, modified Ritchie concentration technique, agar plate culture, and Harada-Mori technique, with the qPCR, there was low agreement, since the estimated kappa index was 0.12 (95% CI: -0.020-0.26). No statistically significant differences were observed in the results obtained in the diagnostic assessment by means of contingency tables and Bayesian analysis (Table 3).

DISCUSSION

In this study, the sensitivity and specificity values obtained by the qPCR were lower than those reported previously, ranging 88.9-100% and 94.8-100%, respectively^{18,19,21,33}. However, the fact that studies reporting 100% sensitivity for the PCR have tested this technique only in samples from people with gastrointestinal symptoms and high parasitic burdens both

for *S. stercoralis* and other pathogens^{18,19,33} is noteworthy; this suggests that results obtained in such studies could neither be extrapolated to the general population nor comparable with data collected in this study.

Regarding other operative features tested compared to qPCR, the diagnostic certainty expressed in the obtained predictive values suggests that the performance of qPCR was higher for identifying people who did not have strongyloidiasis. However, the test presents problems for the detection of true positives. Some studies have reported a good correlation between molecular methods, such as PCR, and conventional parasitology methods used for the detection of *S. stercoralis*, primarily the agar plate culture^{17,33}.

The consensus observed between results from the qPCR and the conventional methods tested in this study was low, coinciding only in the diagnosis of three out of four positive results identified with traditional parasitology techniques (kappa index = 0.12). This may be explained by the fact that, in this study, the qPCR tests detected a larger number of positive samples for *S. stercoralis* than conventional parasitological diagnostic methods. These findings corroborate the results by Moghaddassani et al.¹⁸, who detected five samples with PCR

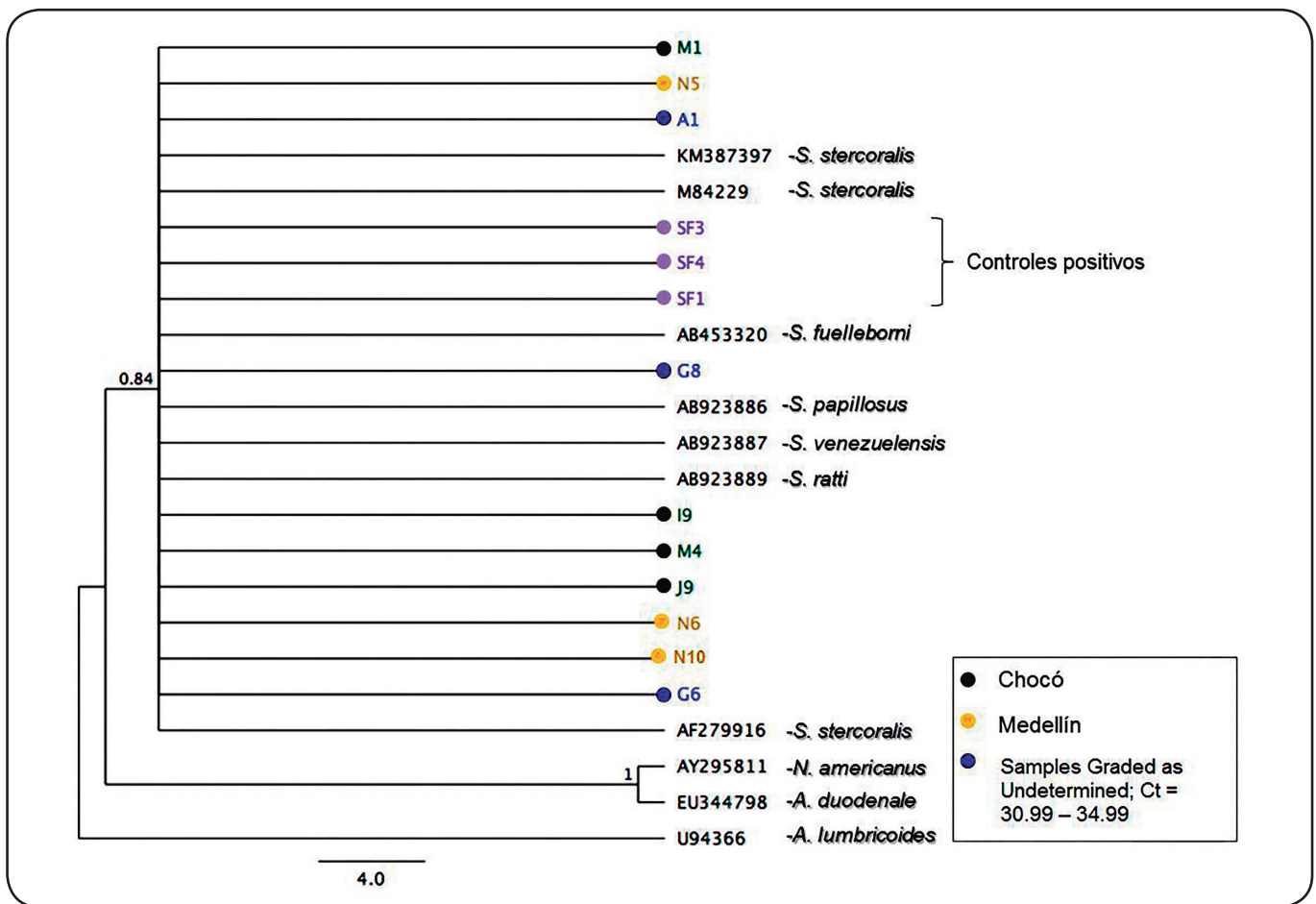


FIGURE 3: Dendrogram obtained by consensus partial sequences of the ribosomal RNA 18S gene from this study reported at the National Center for Biotechnology Information using the Bayesian Inference based on the JC model. The value on the branches indicates the probability *a posteriori*. **RNA:** ribonucleic acid.

that were not previously identified by the reference methods (agar plate culture, direct stool test, and Ritchie concentration technique)¹⁸, suggesting higher efficacy of PCR than that of conventional diagnostic methods for strongyloidiasis, in people with low parasitic burden. The higher number of positive samples by the qPCR can be attributed to the fact that viability and low parasitic burden were not indispensable for the specific detection of this nematode, as opposed to reference diagnostic tests for this parasitic infection, where the larval viability, amount of sample analyzed, and parasitic burden play an essential role in classifying a patient as positive or negative¹².

However, in this particular study, one of the samples that tested positive by conventional methods was not detected by the qPCR, which also influenced the low concordance observed between the different techniques. This finding was similar to that reported by Schar et al.²¹, who reported that the number of samples detected by PCR (38/218) was lower than the amount classified as positive by jointly using the Baermann's method and agar plate culture (41/218)²¹. Likewise, Sharifdini et al.³⁴ reported that their tested PCR failed to detect 13 positive cases by microscopy, suggesting a potential presence of inhibitors in fecal samples.

Furthermore, several factors can explain the occurrence of false-negative results from the qPCR, including preservation, storage, and the presence of inhibitors in fecal samples³⁵. Nsubuga et al.³⁶ analyzed ape fecal samples and reported that the collection and storage temperature of fecal samples played an essential role in DNA recovery³⁶, with lower amounts of genomic DNA isolated from samples collected and stored during warm periods of the year^{37,38}.

Notably, fecal matter is a complex biological sample where the presence of amplification inhibitors might be associated even with an individual's diet, as demonstrated by Monteiro et al.³⁹. They characterized the presence of endogenous inhibitors of PCR and reported that multiple polysaccharides derived from the diet were potent inhibitors to the amplification³⁹. In studies that assessed several DNA isolation protocols in fecal samples depending on the performance and efficiency of the PCR⁴⁰⁻⁴², treatment of samples using physical methods, such as sonication or incubation, at temperatures of 50-100°C using enzymes, such as proteinase K, enhanced the efficiency and performance of the technique. Although some treatments suggested in these investigations were used in the current study as well, additional technical efforts to further optimize the procedures for genomic

DNA isolation from fecal samples are required to improve the performance and efficiency of the tested qPCR.

In this study, primers and probes used for the amplification of the *S. stercoralis* ribosomal RNA 18S gene in the qPCR revealed high species-specificity, evidenced by the analysis of DNA sequences obtained from positive control amplicons and sequenced samples. Although the phylogenetic analyses did not establish a correlation between the analyzed *Strongyloides* specimens and those obtained in this study, the topologies of the phylogenetic trees suggest a close relationship between parasites from Chocó and Medellín and those reported in GenBank. These results support the high specificity of the qPCR method, even highlighting a correlation between samples classified as indeterminate (Ct value = 30.99-34.99) and *Strongyloides* identification, when the parasitic burden was low. Hence, we recommend standardization of a protocol for concentrating the parasitic forms before the isolation of genomic DNA to enhance the concentration of the isolated DNA and the efficiency and linearity of the qPCR.

Concurrently with the results obtained in this study, Schar et al.²¹ reported that the concentration of parasite DNA in the sample plays an essential role in precisely classifying an individual as negative or positive for *S. stercoralis* infection²¹. In addition, the infection stage of a person affects the precise classification, which perhaps directly affects the performance of the qPCR by increasing the Ct value if low concentrations of DNA are isolated from the sample. Hence, it is possible that samples positive for *S. stercoralis* (detected by the qPCR only) were from patients with a chronic parasitic infection characterized by an intermittent excretion of larvae, fluctuations in the parasitic burden, and requiring a multi-sample analysis to detect the parasite using traditional tests, thereby demonstrating the advantage of the qPCR for the detection of low parasitic burdens. Of note, this is suggested because these results were elicited from people who have lived for over 5 years in the outlying neighborhoods of Medellín (La Cruz and Versalles II), but were native to the Chocó and Urabá zones (Department of Antioquia).

In such scenarios, some researchers have implemented Bayesian analyses to estimate the prevalence of *S. stercoralis* and assess the operative characteristics of methods for the detection of this nematode in the absence of a gold standard, suggesting that parameters such as the sensitivity, specificity, and predictive values of the tests evaluated remain unaffected^{43,44}. In this study, the Bayesian analysis of these parameters yielded values analogous to those obtained in the contingency table or 2 × 2 table, assuring with this statistical approach that the tested qPCR method can detect 99% of uninfected people.

Finally, based on the analysis of the sequences obtained for the RNA18S ribosomal gene, Pakdee et al.⁴⁵ reported that *S. stercoralis* populations from different geographical areas (Thailand and Japan) presented genetic divergence⁴⁵. In Colombia, genetic differentiation among *S. stercoralis* populations has not been evaluated at the national level; this is one of the main limitations for evaluating the results obtained in this study, as the genetic diversity of the parasite and how

this could affect the diagnostic sensitivity of the method were not considered. Therefore, other studies that can elucidate the genetic structure of the circulating populations of this parasite are warranted, since they could affect the operational characteristics of the qPCR tested in this study.

In conclusion, this study deduces that although the sensitivity of the tested qPCR was similar to that of the conventional diagnostic methods for strongyloidiasis, it offered the advantage of specifically detecting low concentrations of *S. stercoralis* DNA in fecal samples, especially in cases of chronic phases of the infection. These enhanced features favor the establishment of effective antiparasitic therapies primarily for patients with failed cellular immune responses, whose risk for the development of fatal complications, such as hyperinfection syndrome and dissemination of the parasite, is higher. Of note, the linearity of the method is an essential parameter that should be determined to assess the ability of the test to be used as a quantitative method, as well as to evaluate whether performing a method for the concentration of parasitic forms before genomic DNA isolation increases the likelihood of detecting the parasite in cases of chronic infections owing to low parasitic loads⁴⁶. Furthermore, this aims to evaluate the behavior of the test in different epidemiological and clinical contexts that might provide a better insight into the efficacy of this test.

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Conflict of interest

The authors declare that there is no conflict of interest.

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