Original Article

Scope and limitations of a multiplex conventional PCR for the diagnosis of S. stercoralis and hookworms

Pedro E. Fleitas [a,b,c], Paola A. Vargas [a], Nicolás Caro [a],
M. Cristina Almazan [a,b,c], Adriana Echazú [a,d], Marisa Juárez [a],
Pamela Cajal [a], Alejandro J. Krolewiecki [a,c], Julio R. Nasser [b],
Rubén O. Cimino [a,b,c,*]

[a] Universidad Nacional de Salta, Sede Regional Orán, Instituto de Investigaciones de Enfermedades Tropicales (IIET), Salta, Argentina
[b] Universidad Nacional de Salta, Facultad de Ciencias Naturales, Cátedra de Química Biológica, Salta, Argentina
[c] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), CCT, Salta, Argentina
[d] Universidad Nacional de Salta, Facultad de Ciencias de la Salud, Cátedra de Parasitología, Salta, Argentina

ABSTRACT

Objectives: Describe the diagnostic characteristics of a conventional multiplex PCR for the diagnosis of *S. stercoralis*, *N. americanus* and *Ancylostomas spp*.

Methods: Fecal samples were collected from a cross-sectional study in Orán department, Salta province, Argentina. The stool samples were analyzed using concentration-sedimentation, Harada Mori, McMaster, and Baermann techniques. DNA was extracted from 50 mg fecal sample using the FastPrep® Spin Kit for Soil. Three pairs of primers were used for the amplification of three products of 101, 330, and 577 base pairs (bp) for *S. stercoralis*, *N. americanus* and *Ancylostoma spp*, respectively. The sensitivity and analytical specificity of multiplex PCR were evaluated, as well as the sensitivity and diagnostic specificity, using a composite standard and Bayesian approach.

Results and Conclusions: Multiplex PCR did not present cross-reaction with other intestinal parasites, and the detection limit for multiplex PCR was between 2 and 20 pg of genomic DNA. In addition it presented a diagnostic sensitivity of 97.4% for *S. stercoralis* and 90.3% for hookworms with a specificity of 100% and 87.6%, respectively. PCR identified a higher proportion (p <0.01) of coinfections (15.3%) than microscopic techniques (3.5%). Also, multiplex PCR showed that there was a positive association between *S. stercoralis* and hookworms (odds ratio = 2.12). However, this association was due to *N. americanus* (odds ratio= 3.22), since no association was observed between *S. stercoralis* and *Ancylostoma spp*. Neither was an association observed between the two species of hookworms.

* Corresponding author.
E-mail address: rubencimino@gmail.com (R.O. Cimino).

Introduction

Soil-transmitted helminths (STH) are a major public health problem worldwide, being the most prevalent neglected tropical disease in many poor communities worldwide.[1] STH infections are caused by different species of parasitic worms. *Strongyloides stercoralis* and the hookworms *Necator americanus* and *Ancylostoma duodenale* are STH of significant clinical relevance. Hookworms cause morbidity with chronic intestinal blood loss, iron deficiency anemia, and protein malnutrition affecting growth and cognitive development in children,[2] while *S. stercoralis*, through its ability for autoinfection, can generate chronic infections that can last a lifetime.[3] However, the morbidity of *S. stercoralis* remains unclear.[4]

Mixed infections between hookworms and *S. stercoralis* are common in endemic areas.[5] This may be because both share the same infection route.[6] However, these parasites have different life cycles, and while the diagnosis of *S. stercoralis* depends on the identification of larvae, the diagnosis of hookworm, and other STH, is mainly based on the identification of eggs. The Kato-Katz method, which is based on egg quantification, is the most commonly used technique for assessing STH prevalence and intensity of infection in epidemiological surveys.[7] Other methodologies include the concentration-sedimentation technique, and Mc Master, which, like Kato-Katz, are cheap and simple methods. However, the recommended diagnostic methods for *S. stercoralis* are the Baermann, agar plate, and Harada-Mori techniques, since they allow visualization of the larvae. These methods are laborious and require specialized technicians who can morphologically differentiate *S. stercoralis* larvae from hookworm larvae.[8] Therefore, such methods are not suitable for routine use in many laboratories. In turn, the diagnosis of hookworms is not as challenging as *S. stercoralis*. However, microscopy of fecal samples should be done rapidly because hookworm eggs disintegrate in fecal matter,[9] and the identification of the different hookworm species cannot be done through the observation of eggs. These difficulties may underestimate the true prevalence of these parasites and the prevalence of mixed infections.

Interventions for the control of STH require a sound and timely assessment of the epidemiological situation based on sensitive and specific diagnostic methods. Herein we describe a conventional multiplex PCR assay, which can simultaneously detect three species of STH, namely *N. americanus, Ancylostoma spp.* (including *A. caninum, A. ceylanicum* and *A. duodenale*), and *S. stercoralis*, which as we have recently shown, correlate with their distribution.[10]

Material and methods

Study population

A sample size of 258 individuals was calculated using Burderer's formula,[11] assuming a prevalence of 30% for *S. stercoralis,*[12] an anticipated PCR sensitivity of 72%,[13] an absolute precision of 10%, and a confidence interval of 95%.

Fecal samples were collected from a cross-sectional study in Orán department, Salta province, Argentina. Eligible individuals were children and adults assigned to a health intervention program carried out by the teams of the Universidad Nacional de Salta.

The study was approved by the Bioethics Committee of the Faculty of Health Sciences at the Universidad Nacional de Salta (Res-CDN° 417/15). All participants were informed about the purpose and procedures of the study. All individuals infected with STH were treated with a combination of single dose albendazole (400 mg) and ivermectin (200 µg/kg).

Microscopy

A single stool sample was collected from each participant in sterile containers without preservatives. Stool samples were dated, coded and then analyzed within 24 hours of collection using concentration-sedimentation and McMaster techniques for egg detection and Baermann and Harada-Mori techniques for larvae detection.[14] Any sample that tested positive for at least one technique was considered positive for that particular species of STH.

DNA extraction and PCR

DNA was extracted from 50 mg fecal sample using the FastPrep® Spin Kit for Soil (MP Biomedicals, Santa Ana, USA) according to the manufacturer's instructions for all parasites.[15] All samples were processed in Salta, Argentina.

Specific primers for detecting *N. americanus* and *S. stercoralis* were designed by other authors.[16,17] The primers for detecting *Ancylostoma spp* were designed using Primer3 (v.0.4.0) software[18] from ribosomal DNA region of *A. duodenale* (GenBank Accession N°: EU344797.1). The forward primer was CAT GAA TGC CGC CTT ACT GC and the reverse primer was AAG TTC AGC GGG TAG TCA CG. The three primer pairs allow the amplification of three products of 101, 330 and, 577 base pairs (bp) for *S. stercoralis, N. americanus* and *Ancylostoma spp,* respectively.

PCR reaction mixture consisted of 10 µl DNA template, 0.125 µl GoTaq® polymerase (Promega, Madison, USA), 5 µl 10x GoTaq® buffer (Promega, Madison, USA), 0.5 µl dNTP (10 mM), 0.2 µM *N. americanus* primers, 0.2 µM *S. stercoralis* primers, 0.3 µM *Ancylostoma spp* primers, and 5.875 µl MiliQ water. PCR conditions consisted of an initial denaturation at 95° C for 3 min, followed by 35 cycles of 95° C for 45 s, 61° C for 1 min, and 72° C for 45 s, and a final elongation was performed at 72° C for 5 min. For each PCR, a negative control (MiliQ water) was run together. PCR products were analyzed by electrophoresis on 1.5% agarose gels, stained with GelRed® 1X (Biotium, San Francisco, USA), and visualized on a UV transilluminator.

Sequencing of PCR products

Five PCR products from each helminth were randomly chosen. The PCR products were recovered from the 1.5% agarose gels using the Puro Gel Extraction kit (PB-L Productos Bio-Logicos, Argentina) and then sequenced at the Instituto Nacional de Tecnología Agropecuaria (INTA, Castelar).

The sequences obtained were aligned with the MEGA X software,[19] and an alignment with the NCBI database was performed with the BLAST software.[20]

Analytical specificity and limit of detection of multiplex PCR

The multiplex PCR was evaluated for specificity using fecal samples with humam intestinal parasites such as *Ascaris lumbricoides, Trichuris trichiura, Giardia intestinalis, Hymenolepis diminuta, Hymenolepis nana, Enterobius vermicularis,* and *Entamoeba coli*. In turn, to determine the limit of DNA detection, 10-fold serial dilutions of a mixture of three positive samples of *S. stercoralis, N. americanus,* and *Ancylostoma spp* were performed. The mixture of DNA from *Ancylostoma spp., N. americanus,* and *S. stercoralis* was performed by measuring the concentration of each DNA extraction individually with the NanoDrop 2000 spectrophotometer (Thermo Fisher, Waltham, United States). Each DNA extraction was then diluted to a concentration of 60 ng/μl with ultrapure water and mixed in equal parts, obtaining a final concentration of 20 ng/μl of each DNA extract in the mixture.

Statistical analysis

Diagnostic precision parameters for multiplex PCR and the combination of the four microscopic techniques were evaluated by two different approaches. First, multiplex PCR was compared with a composite standard, consisting of the result of multiplex PCR and microscopic techniques. In this case, an individual was considered positive if the presence of an egg or larva was observed by any of the microscopic techniques and/or multiplex PCR. All these analyses were carried out with R software,[21] and statistical significance was assessed by McNemar test. In the second approach, since microscopic methods are imperfect standards due to false negative results and less than perfect sensitivity and specificity, a Bayesian approach was used to estimate diagnostic parameters in the absence of a gold standard. It was assumed that PCR follows a biological process that is different from microscopic techniques,[22] and model 101 of the online platform Modeling of Infectious Disease Center (MICE) was used.[23] For this, the sample was randomly divided into two subpopulations with 65% of the data in one and 35% in the other. In this case, a significant difference was considered when there was no overlap of the confidence intervals.

To measure the correlation between *S. stercoralis* and hookworms, logistic regression was performed with the R software.[21]

Results

Mixed infections and asociation between helminth species

Out of 287 stool samples collected in the province of Salta, 13% (38/287) and 19% (54/287) were positive for hookworms and *S. stercoralis* by microscopic techniques, respectively. Multiplex PCR were positive in 28% (80/287) and 39% (113/297) for hookworms and *S. stercoralis*, respectively (Fig. 1). In addition, multiplex PCR identified a higher proportion (p <0.01) of
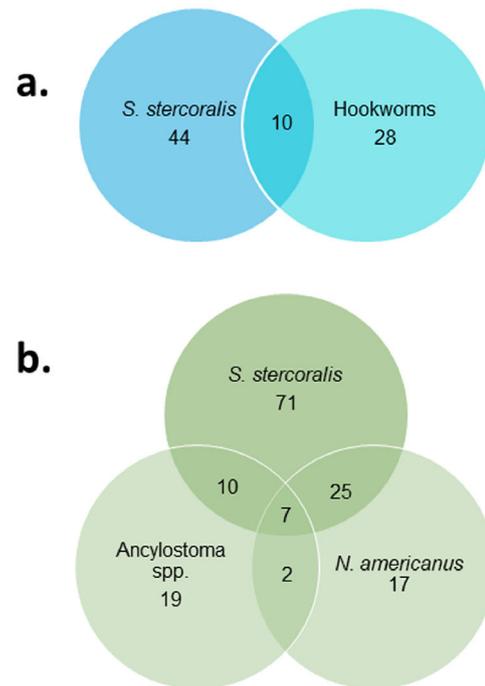

Fig. 1 – Venn diagram for those infected by *S. stercoralis* and hookworms. (a) Microscopic techniques. (b). Multiplex PCR.

coinfections (15.3%; 44/287) than microscopic techniques (3.5%; 10/287).

There was a positive association between *S. stercoralis* and hookworms (odds ratio= 2.12 CI 95%: 1.25 − 3.58) with multiplex PCR. However, this association was due to *N. americanus* (odds ratio= 3.22 CI 95%: 1.72 − 6.04), since no association was observed between *S. stercoralis* and *Ancylostoma spp.* (odds ratio= 1.29 CI 95%: 0.65 − 2.57). Neither was an association observed between the two species of hookworms (odds ratio= 1.53 CI 95%: 0.67 − 3.47). On the other hand, no significant association was observed between *S. stercoralis* and hookworms when analyzing the results of microscopic tests.

Sensitivity and analytical specificity of multiplex PCR

Multiplex PCR for the diagnosis of *Ancylostoma spp., N. americanus,* and *S. stercoralis* was able to specifically amplify the DNA of the three helminths with no cross-reaction with other intestinal parasites (Fig. 2). In addition, the sequences analyses of the PCR products revealed that the PCR products of *S. stercoralis* showed 100% homology with the 18S sequence of *S. stercoralis* (GenBank: AF279916), the PCR products of *N. americanus* presented two different sequences that presented 100% and 98.9% homology with the sequence ITS1 of *N. americanus* (GenBank: AJ001680.1), and the PCR products of *Ancylostoma spp* presented two different sequences, one presented 100% homology with the ribosomal sequences of *A. duodenale* (GenBank: EU344797.1) and the other 99.8% homology with *A. duodenale* (GenBank: EU344797.1 and MK271367.1), 98.7% with *A. caninum* (GenBanK: KP844730.1), and 98.5% with *A. ceylanicum* (GenBank: LC036567). This indicates that multiplex PCR is specific for *S. stercoralis, N. americanus,* and *Ancylostoma spp*.
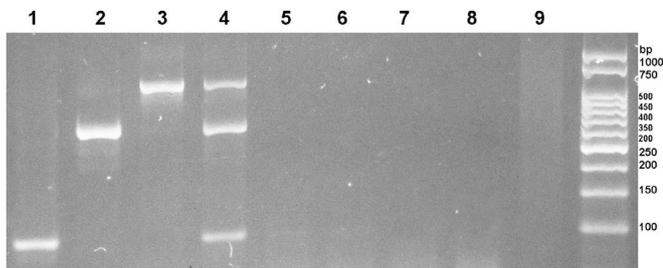
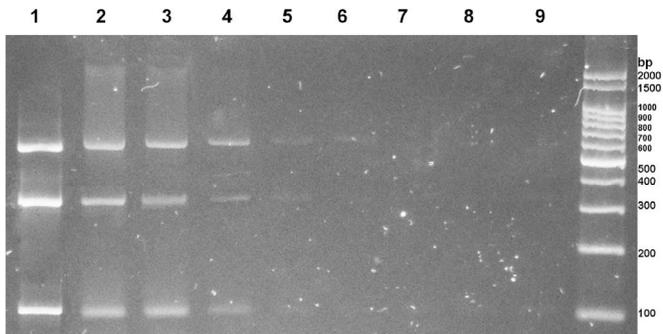**Fig. 2 – Analytical specificity of multiplex PCR.**

Fig. 3 – Analytical sensitivity of multiplex PCR.

Sensitivity analysis revealed that the detection limit for multiplex PCR was between 2 and 20 pg of genomic DNA from each helminth of interest (Fig. 3).

In lanes 1, 2, and 3 fecal samples positive for *S. stercoralis, N. americanus* and *A. duodenale,* respectively. In lane 4, a positive stool for *S. stercoralis, N. americanus* and *A. duodenale.* In lanes 5, 6, 7, 8, and 9 fecal samples with *A. lumbricodes, T. trichiura* and *H. nana, G. intestinalis* and *H. diminuta, E. vermicularis* and *E. coli,* and *A. lumbricodes* and *T. trichiura.*

Successive 1/10 dilutions of a *mixture of DNA* from Ancylostoma spp., *N. americanus,* and *S. stercoralis* are observed in lanes 1 to 9, with an initial concentration of 20 ng/μl.

Diagnostic evaluation of multiplex PCR

The diagnostic sensitivity of multiplex PCR for *S. stercoralis* and hookworms was significantly higher using the composite standard (McNemar test p <0.01), and the Bayesian approach. In turn, the diagnostic specificity of multiplex PCR was not significantly different from microscopic techniques using the composite standard (McNemar test p >0.05), and the bayesian approach (Table 1).

Discussion

As deworming campaigns expand and new targets are outlined for the control of STH as a public health problem, diagnostic tools with the appropriate sensitivity, specificity and ability of species identification are needed to meet those targets.[24] The lack of accurate diagnostic tools is also an important consideration for mapping the epidemiological situation of STH. Limitations in diagnostic techniques have led to the exclusion, for years, of *S. stercoralis* in the WHO strategies against STH. However, the new WHO report incorporates a control strategy for *S. stercoralis* in school-age children.[24] This new scenario implies that rapid and sensitive diagnostic methods are needed to identify risk areas for *S. stercoralis*, which can also be risk areas for hookworms.[25] Therefore, in this study, a diagnostic strategy that would allow simultaneous identification of *S. stercoralis* infection and the two species of hookworms was approached. Multiplex PCR was able to specifically detect the three species of interest even in the presence of other helminths with the advantage of being able to differentiate between hookworm species. This implies that the analytical specificity of the technique would not be compromised in endemic areas where different species of helminths coexist. However, the detection limit of multiplex PCR (20 pg/μl) was shown to be three orders of magnitude higher than the limit of detection (fg/μl) of multi-parallel qPCR for *S. stercoralis* and hookworms.[15] This demonstrates a lower analytical sensitivity of conventional PCR compared to qPCR. However, it was shown that, in areas of high prevalence, conventional PCR has a higher sensitivity than microscopic techniques with the same specificity. It should be noted that in the absence of a reference technique, the Bayesian approach allows an estimation of the true sensitivity and specificity of a new diagnostic technique.[23] Through this approach, it was observed that the sensitivity of multiplex PCR for *S. stercoralis* was higher than that reported by Buonfrate et al.[13] and was similar to the sensitivity and specificity of a urine PCR technique evaluated in the same geographical area.[12] On the other

Table 1 – Diagnostic sensitivity and specificity of microscopic techniques and multiplex PCR.					
Strongyloides stercoralis		Sensitivity (95% CI)	Specificity (95% CI)	Positive predictive value (95% CI)	Negative predictive value (95% CI)
Composite standard	Microscopic	46.6% (37.0-56.1)	100% (99.7-100)	100% (99.1-100)	73.4% (67.5-79.3)
	PCR multiplex	97.4% (94.0-100)	100% (99.7-100)	100% (99.6-100)	98.3% (96.1-100)
Bayesian approach	Microscopic	68.9 (41.0-99.9)	99.0 (96.1100)	96.1 (85.3-100)	89.8 (69.6-100)
	PCR multiplex	97.4 (88.1-100)	81.2 (69.5-99.9)	64.8 (39.3-99.8)	98.9 (93.7-100)
Hookworms					
Composite standard	Microscopic	43.7 (32.7-54.7)	100 (99.7-100)	100 (98.7-100)	80.3 (75.2-85.5)
	PCR multiplex	91.25 (85.7-98.2)	100 (99.7-100)	100 (99.4-,100)	96.6 (93.9-,99.3)
Bayesian approach	Microscopic	58.8 (31.9-99.8)	98.1 (94.4-100)	88.6 (66.4-100)	90.7 (73.1-100)
	PCR multiplex	90.3 (71.2-100)	87.6 (77.2-100)	64.1 (31.8-99.9)	97.6 (88.2-100)

hand, the sensitivity and specificity of multiplex PCR for hookworms are similar to that reported for qPCR.[26]

In addition, a single multiplex PCR assay had a sensitivity that can only be achieved with microscopic methods if several assays are performed with numerous samples. These results show that conventional PCR provides sufficient information for estimating prevalence, and identifying priority areas for control programs, where infection levels are typically high. Nonetheless, techniques such as real-time PCR are more suitable to evaluate elimination of transmission, clinical trials, and mathematical modeling studies of transmission dynamics.[27,28]

Mixed infection is an important factor to consider before implementing mass drug administration, and selecting the appropriate antiparasitic drugs.[29] In turn, infection with one helminth can alter the intensity of infection by another helminth.[30] A significantly higher proportion of mixed infections was detected by multiplex PCR. In this study, the most frequent mixed infections were *S. stercoralis* and *N. americanus*, and *S. stercoralis* and *Ancylostoma spp.* This may be due to a higher prevalence of *S. stercoralis* in the area. In line with other reports,[31,32] we also found a positive association between *S. stercoralis* and hookworms. However, this association only applied to *N. americanus*, and that no correlation was found between the two species of hookworms. Subsequent studies in areas with a high prevalence of hookworm and *S. stercoralis* are necessary to understand whether these results are specific to this area or due to distinctive features of different species of hookworms and potential interaction between the different species of hookworms. Also, the correlation analysis was significant only with the multiplex PCR results, noting that there was greater correlation between *S. stercoralis* and hookworms when high sensitivity diagnostic methods were used.[10] At the same time, the correlation between *S. stercoralis* and hookworms highlights the importance of generating control strategies that include both to allow a more efficient use of resources.

Certain limitations must be taken into account when interpreting this study. First, our multiplex PCR only provides qualitative results, being unable to measure the intensity of the infection. This presents an important limitation in the case of hookworm, since the number of excreted eggs cannot be known; however, it is of no importance for *S. stercoralis*, whose intensity of infection cannot be clearly measured,[12,33] so a quantitative measurement does not represent an advantage in the latter case. In addition, it should be noted that although there is a relationship between the qPCR result and the intensity of infection, this relationship is neither simple nor fully understood.[33] Moreover, the correlation between eggs per gram and the respective qPCR results is lower in hookworms than in *T. trichiura* y *A. lumbricoides*.[28] Second, it was not possible to evaluate whether the primers of the genus Ancylostoma had the ability to differentiate between *Ancylostoma ceylanicum* and *Ancylostoma duodenale*. However, as no cases of *A. ceylanicum* have been reported in northern Argentina,[34] it is likely that all the *Ancylostoma* cases detected were due to *A. duodenale*. Further investigations will elucidate whether *Ancylostoma* primers can amplify the DNA of various *Ancylostoma* species.

Finally, it can be affirmed that multiplex PCR presented a superior performance to microscopic techniques in high prevalence areas. This is an additional tool to detect several species of parasites simultaneously in a large population and to evaluate the epidemiological situation of the region in order to design deworming strategies.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

REFERENCES

1. Scolari C, Torti C, Beltrame A, et al. Prevalence and distribution of soil-transmitted helminth (STH) infections in urban and indigenous schoolchildren in Ortigueira, State of Parana, Brasil: implications for control. Trop Med Int Heal. 2000;5:302–7. https://doi.org/10.1046/j.1365-3156.2000.00549.x.

2. Sakti H, Nokes C, Hertanto WS, et al. Evidence for an association between hookworm infection and cognitive function in Indonesian school children. Trop Med Int Heal. 1999;4:322–34. https://doi.org/10.1046/j.1365-3156.1999.00410.x.

3. Schär F, Odermatt P, Khieu V, et al. Evaluation of real-time PCR for Strongyloides stercoralis and hookworm as diagnostic tool in asymptomatic schoolchildren in Cambodia. Acta Trop. 2013;126:89–92. https://doi.org/10.1016/j.actatropica.2012.12.012.

4. Tamarozzi F, Martello E, Giorli G, et al. Morbidity associated with chronic strongyloides stercoralis infection: a systematic review and meta-analysis. Am J Trop Med Hyg. 2019;100:1305–11. https://doi.org/10.4269/ajtmh.18-0895.

5. Echazú A, Bonanno D, Juarez M, et al. Effect of poor access to water and sanitation as risk factors for soil-transmitted helminth infection: selectiveness by the infective route. PLoS Negl Trop Dis. 2015;9:1–14. https://doi.org/10.1371/journal.pntd.0004111.

6. Strunz EC, Addiss DG, Stocks ME, Ogden S, Utzinger J, Freeman MC. Water, sanitation, hygiene, and soil-transmitted helminth infection: a systematic review and meta-analysis. PLoS Med. 2014;11. https://doi.org/10.1371/journal.pmed.1001620.

7. Knopp S, Salim N, Schindler T, et al. Diagnostic accuracy of Kato-Katz, FLOTAC, baermann, and PCR methods for the detection of light-intensity hookworm and Strongyloides stercoralis infections in Tanzania. Am J Trop Med Hyg. 2014;90:535–45. https://doi.org/10.4269/ajtmh.13-0268.

8. Becker SL, Piraisoody N, Kramme S, et al. Real-time PCR for detection of Strongyloides stercoralis in human stool samples from C??te d'Ivoire: diagnostic accuracy, inter-laboratory comparison and patterns of hookworm co-infection. Acta Trop. 2015;150:210–7. https://doi.org/10.1016/j.actatropica.2015.07.019.

9. Dacombe RJ, Crampin AC, Floyd S, et al. Time delays between patient and laboratory selectively affect accuracy of helminth diagnosis. Trans R Soc Trop Med Hyg. 2007: 140–5. https://doi.org/10.1016/j.trstmh.2006.04.008.

10. Fleitas PE, Travacio M, Martí-Soler H, Socías ME, Lopez WR, Krolewiecki AJ. The Strongyloides stercoralis-hookworms association as a path to the estimation of the global burden of strongyloidiasis: a systematic review. PLoS Negl Trop Dis. 2020;14:e0008184. https://doi.org/10.1371/journal.pntd.0008184.

11. Malhotra R, Indrayan A. A simple nomogram for sample size for estimating sensitivity and specificity of medical tests. Indian J Ophthalmol. 2010;58:519. https://doi.org/10.4103/0301-4738.71699.

12. Krolewiecki AJ, Koukounari A, Romano M, et al. Transrenal DNA-based diagnosis of Strongyloides stercoralis (Grassi, 1879) infection: bayesian latent class modeling of test accuracy. PLoS Negl Trop Dis. 2018;12:e0006550. https://doi.org/10.1371/journal.pntd.0006550.

13. Buonfrate D, Requena-Mendez A, Angheben A, et al. Accuracy of molecular biology techniques for the diagnosis of Strongyloides stercoralis infection—A systematic review and meta-analysis. PLoS Negl Trop Dis. 2018;12:e0006229. https://doi.org/10.1371/journal.pntd.0006229.

14. Garcia LS. Diagnostic Medical Parasitology. Manual of Commercial Methods in Clinical Microbiology. Washington, DC, USA: ASM Press; 2014. p. 274–305.

15. Cimino RO, Jeun R, Juarez M, et al. Identification of human intestinal parasites affecting an asymptomatic peri-urban Argentinian population using multi-parallel quantitative real-time polymerase chain reaction. Parasit Vectors. 2015;8:380. https://doi.org/10.1186/s13071-015-0994-z.

16. Phuphisut O, Yoonuan T, Sanguankiat S, et al. Triplex polymerase chain reaction assay for detection of major soil-transmitted helminths, Ascaris lumbricoides, Trichuris trichiura, Necator americanus, in fecal samples. Southeast Asian J Trop Med Public Health. 2014;45:267–75.

17. Verweij JJ., Canales M., Polman K., et al. Molecular diagnosis of Strongyloides stercoralis in faecal samples using real-time PCR. 2009:342–6. https://doi.org/10.1016/j.trstmh.2008.12.001.

18. Untergasser A, Cutcutache I, Koressaar T, et al. Primer3-new capabilities and interfaces. Nucleic Acids Res. 2012;40:1–12. https://doi.org/10.1093/nar/gks596.

19. Kumar S, Stecher G, Li M, Knyaz C, Tamura K, MEGA X. Molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 2018;35:1547–9. https://doi.org/10.1093/molbev/msy096.

20. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215:403–10. https://doi.org/10.1016/S0022-2836(05)80360-2.

21. Core Team R. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Viena, Austria. http://www. R-project. org; 2017.

22. Knopp S, Salim N., Schindler T., et al. Diagnostic accuracy of Kato − Katz, FLOTAC, Baermann, and PCR methods for the detection of light-intensity hookworm and Strongyloides stercoralis infections in Tanzania. 2014;90:535−45. https://doi.org/10.4269/ajtmh.13-0268.

23. Lim C, Wannapinij P, White L, et al. Using a web-based application to define the accuracy of diagnostic tests when the gold standard is imperfect. PLoS One. 2013;8:1–8. https://doi.org/10.1371/journal.pone.0079489.

24. World Health Organization. 2030 Targets for Soil-Transmitted Helminthiases Control Programmes.Licence:CC BY-NC-SA 3.0 IGO. WHO; 2019.

25. Forrer A., Khieu V., Schär F., et al. Strongyloides stercoralis and hookworm co-infection: spatial distribution and determinants in Preah Vihear Province, Cambodia. Parasites and Vectors. 2018;11:1−13. https://doi.org/10.1186/s13071-017-2604-8.

26. Schär F, Trostdorf U, Giardina F, et al. Strongyloides stercoralis: global distribution and risk factors. PLoS Negl Trop Dis. 2013;7:e2288. https://doi.org/10.1371/journal.pntd.0002288.

27. Krolewiecki AJ. Misconceptions and paradoxes in soil-transmitted helminthiases control as a public health problem. PLoS Negl Trop Dis. 2018;12:e0006672. https://doi.org/10.1371/journal.pntd.0006672.

28. Papaiakovou M, Littlewood DTJ, Gasser RB, Anderson RM. How qPCR complements the WHO roadmap (2021−2030) for soil-transmitted helminths. Trends Parasitol. 2021;xx(xx):1–11. https://doi.org/10.1016/j.pt.2021.04.005.

29. Hotez P. Enlarging the "audacious goal": elimination of the world's high prevalence neglected tropical diseases. Vaccine. 2011;29(SUPPL. 4):D104−10. https://doi.org/10.1016/j.vaccine.2011.06.024.

30. Fleming FM, Brooker S, Geiger SM, et al. Synergistic associations between hookworm and other helminth species in a rural community in Brazil. Trop Med Int Heal. 2006;11:56–64. https://doi.org/10.1111/j.1365-3156.2005.01541.x.

31. Becker SL, Sieto B, Silué KD, et al. Diagnosis, clinical features, and self-reported morbidity of Strongyloides stercoralis and hookworm infection in a co-endemic setting. PLoS Negl Trop Dis. 2011;5. https://doi.org/10.1371/journal.pntd.0001292.

32. Blackwell AD, Martin M, Kaplan H, Gurven M. Antagonism between two intestinal parasites in humans: the importance of co-infection for infection risk and recovery dynamics. Proc R Soc B. 2013;280:20131671. https://doi.org/10.1098/rspb.2013.1671.

33. Papaiakovou M, Gasser RB, Littlewood DTJ. Quantitative PCR-based diagnosis of soil-transmitted helminth infections: faecal or fickle? Trends Parasitol. 2019;35:491–500. https://doi.org/10.1016/j.pt.2019.04.006.

34. Papaiakovou M, Pilotte N, Grant JR, et al. A novel, species-specific, real-time PCR assay for the detection of the emerging zoonotic parasite Ancylostoma ceylanicum in human stool. PLoS Negl Trop Dis. 2017;11:e0005734. https://doi.org/10.1371/journal.pntd.0005734.