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Research Article
Simultaneous Detection of *Ascaris lumbricoides*, *Trichuris trichiura* and *Strongyloides stercoralis* using Modified Stool DNA Extraction Method

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Abstract

Background and Objective: Soil Transmitted Helminth (STH) infection is diagnosed by using a conventional method like microscopy, egg numeration assay, serology based assay and egg culture. Polymerase Chain Reaction (PCR) has advantages over the traditional microscopy method as they are highly sensitive and specific. However, disruption of the hard eggs shell covering the STHs eggs may hamper the DNA extraction process. This study was carried out to compare the sensitivity of microscopy and conventional PCR techniques to detect *A. lumbricoides, T. trichiura* and *S. stercoralis* using modified DNA extraction technique in stool samples. **Material and Methods:** Prior to DNA extraction, the isolated eggs were subjected to floatation and sedimentation techniques were briefly boiled followed by freeze-thaw cycles. Around eight (n = 8) stool samples positive for STH were chosen randomly by microscopy were proceeded by PCR technique for identification of *A. lumbricoides*, *T. trichiura* eggs and *S. stercoralis* larvae. **Results:** The presence of *A. lumbricoides* and *T. trichiura* eggs were detected in all eight stool samples (100%) by microscopy examination while only six samples showed positive PCR amplification (sensitivity: 75%) for *A. lumbricoides* and two samples show positive PCR amplification (sensitivity:20%) for *T. trichiura*. On the other hand, there are no *S. stercoralis* were detected in all stool samples. **Conclusion:** Microscopy was proven to be rapid and inexpensive compare to PCR however, the additional study needs to standardize DNA extraction and conventional PCR protocol in order to increase diagnosis.

Key words: DNA extraction, PCR assay, STHs infection, sensitivity, modified DNA methods, egg pre-treatment

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Statistics from the World Health Organization's (WHO) indicated that around 1.5 billion people are infected with Soil Transmitted Helminth (STH) infection and in need of treatment[1]. STH is a communicable disease which has a low number of cases can not be highly prevalent but can cause serious health issue worldwide[2]. Systemic review conducted in Asia showed approximately 70% of the STHs infection is attributed to the moist and tropical climatic conditions or poor living conditions which aid in worm survival and transmission[3].

Ascaris lumbricoides (roundworm) is the most common STHs infection with a prevalence of 15% as compared to other helminths such as *Necator americanus* (5%), *Ancylostoma* spp. (2.5%), *Shistoma* spp. (1.6%) and *Strongyloides stercoralis* (1%)[2]. However, according to a study carried out in rural and remote West Malaysia, *T. trichiura* has the highest prevalence of STHs infection (66.8%) followed by *A. lumbricoides* (38.5%) and hookworm infection (12.8%). However, the most potent co-infection observed was the combination of *T. trichiura* and *A. lumbricoides* (30.6%) and the infection leads to nutritional deficiency and in severe cases death has been reported[4,5]. As for *Strongyloidiasis*, at least 30-100 million cases were reported with this infection in Southeast Asia, South America and Africa[6]. In Malaysia, the prevalence rate of *S. stercoralis* infection among the rural communities ranges between 1.2-1.7% as well other countries such as Cambodia (10.3-21%), China (11.7%) and Japan (5-10%)[7].

In 2012, WHO had set a worldwide goal to eradicate STHs infection as a public health problem by 2020, achieving 75% of Mass Drug Administration (MDA) coverage in affected area[8]. However, MDA effort has only successfully implemented in 44.7% school children and 51.4% in preschool-children globally[9]. Therefore, better diagnosis is needed to monitor infection level, response to treatment as well as determining the endpoint of MDA.

Currently, the diagnosis is focused on the molecular-based approach such as Polymerase Chain Reaction (PCR) as this method is highly sensitive and specific[10]. Previous studies have proven that microscopy has a sensitivity of 70-88% whilst molecular test has a sensitivity of 85-100% for *A. lumbricoides* and 88% versus 100% for *T. trichiura* [11]. As there is no gold standard for STH detection, most of the studies compare the result of a few test to evaluate the sensitivity and specificity of the test[12]. Hence this study was carried out to compare the sensitivity of microscopy and conventional PCR techniques to detect *A. lumbricoides*, *T. trichiura* and *S. stercoralis* using modified DNA extraction technique in stool samples.

MATERIALS AND METHODS

Sample collection and preparation: Stool samples used were previously collected from Kampung Orang Asli Kachau Luar, Semenyih, Malaysia. The study was carried out in Parasitology Laboratory at Institute of Medical Science Technology, Universiti Kuala Lumpur (UniKL) from January, period from when it was started. The stool samples were preserved in 70% alcohol and eight (n = 8) stool samples were choose randomly to be used in this study. About 10 g of fecal samples were homogenized in 5 mL of distilled water by using a wooden tongue depressor. Then, the mixture was filtered three times by using wet cotton cheesecloth. Centrifuge the sample at 3000 rpm for ten minutes to push the eggs to the bottom and the supernatant was removed.

Eggs isolation and larvae separation by using flotation and sedimentation technique: The egg isolation technique was modified as described by Mehru Nisha *et al.*[13]. For *Ascaris* spp. and *Trichuris* spp., the pellet was suspended in salt and sugar solution and was left to stand for 10 min at room temperature to allow the eggs to float at the surface as well as the debris to sink at the bottom. After that, centrifuge the solution at 25,000 rpm for 5 min. Collect the eggs from the supernatant and transferred it to 2 mL of microcentrifuge tube for further use. For *Strongyloides stercoralis*, the Formalin Ethyl Acetate Concentration (FEAC) technique was carried out to separate larvae from debris. Around 3-5gram of stool sample was mixed with 7 mL of distilled water instead of 10% formalin and passed through of two layers of dampened gauze into a clean centrifuge tube. The supernatant was mixed with 2-3 mL of ethyl acetate and centrifuged for 2 min at 12,000 rpm. Four layers of separation appeared after centrifuged which consist of ethyl acetate at the top, the sec layer is a plug of debris, third is a clear layer of distilled water and sediment with helminths at the bottom.

Microscopy examination: A small volume of stool sample (n = 8) after flotation fluid and sediment was smeared onto a slide and observed under a microscope.

Egg disruption method: Initial freezing followed by boiling method was chosen as it is a simple, rapid, economical and minimally strenuous method to crack the eggs and release the genomic DNA for the qualitative study for diagnosis[13]. First, 200 µL of egg suspension and 400 µL of Buffer ATL (QIAamp DNA Mini Kit) were aliquoted into a new 2 mL microcentrifuge tube. Seven cycles of freezing the eggs rapidly in liquid nitrogen for 10 min and to heating at 100°C

Table 1: Primer for *Ascaris lumbricoides*, *Trichuris trichiura* and *Strongyloides stercoralis*[18,19]

Species	Primer sequence	Amplicon size	Annealing size (Ta)
Ascaris lumbricoides	5′CCAGCTGACGCACTCGCTTGG 3′ 3′ATGGTTGAGGTCTCCGTATGTG 5′ β-tubulin gene isotype 1	158-bp fragment	59°C
Trichuris trichiura	5′GGCTAAAGGGCACTATACG3′ 3′GGAAAGCGTAGGCATGTCG5′ β-tubulin gene isotype 1	163-bp fragment	59°C
Strongyloides stercoralis	5′GAATTCCAAGTAAACGTAAGTCATTAGC3′ 3′-TGCCTCTGGATATTGCTCAGTTC5′ 18S rRNA	101-bp fragment	55°C

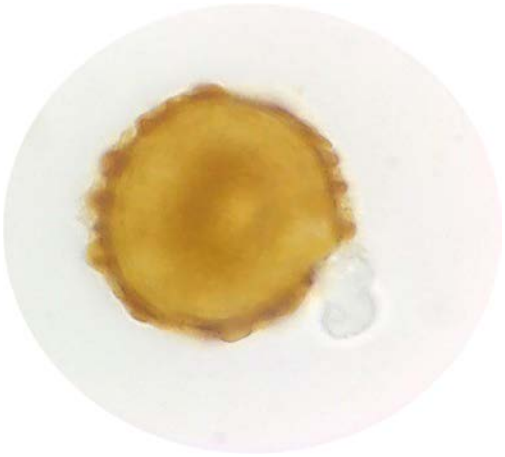

Fig. 1: Egg lysis after 7 cycle of freeze/thaw under at 100× magnification (Mehru Nisha *et al*.[13])

for another 10 min was used routinely to lyse the eggs[14]. During the freeze/thaw cycle, the samples were vortexed occasionally (Fig. 1).

Conventional PCR examination: The same eight stool samples from flotation techniques were subjected to conventional PCR procedure. In order to extract the DNA efficiently, the eggs undergone a disruption method to lyse the wall of the eggs was used for nucleic acids isolation[15]. For DNA extraction commercial mini kit was used to extract the DNA from the eggs. There were four major steps following the manufacture's protocol which are lysis procedure followed by binding DNA to silica membrane, washing and elution. Next, the Quantification of nucleic acids was performed to determine their purity and concentration for optimum performance of PCR. The primer sequence and PCR set up are as in Table 1-3.

DNA extraction: QIAamp DNA Mini Kit (Qiagen, Germany) contain silica membrane-based purification of up to 30 µg of genomic, bacterial, viral and parasite DNA. DNA extraction was used as per manufacture's protocol with modifications.

Table 2: PCR master mix

PCR reaction mixture	Positive control	Samples
2x PCR master mix solution (i-pfu)	10	10
Template DNA	2	5
Primer (F: 10 pmol µL)	1	1
Primer (R: 10 pmol µL)	1	1
Distilled water	6	3
Total	20 µL	20 µL

Table 3: PCR setup

Steps	Temperature (°C)	Time	Cycle
Initial denaturation	98	10 sec	1
Cyclic step			
Denaturation	98	1 sec	40
Annealing	According to each primer set	5 sec	
Elongation	72	15 sec*	
Final elongation	72	5 min	1

After the eggs disruption method, the sample was vortex for 15 sec before centrifugation at full speed to pellet the stool particle.

Then, 1.5 µL of the proteinase K was pipetted into another 1.5 µL micro centrifuge tube. About 200 µL of the supernatant of the processed sample was added with proteinase K and addition of 200 µL of Buffer AL and vortex for 15 sec to mix the contents thoroughly. The samples were incubated at 55°C for 30 min to allow lysis and digestion of protein took place[15]. After 30 min, the lysate was added with pre-cooled ethanol (96%) to precipitate the DNA[16]. The sample was vortexed and centrifuged before transfered to QIAamp spin column for binding of DNA to replace with the filter membrane. For the washing step, the column was washed with Buffer AW1 (500 µL) to wash away cell debris and other particles followed by Buffer AW2 (500 µL) to purify the DNA. Lastly, 25 µL AE buffer was transfered into the column and incubated for 20 min before centrifugation at full speed to elute the DNA[17].

DNA concentration: Nanophotometer was used to determine the concentration and purity of DNA by measuring absorbance at 260/280 nm and absorbance at 260/230nm.

Conventional PCR: In this study, conventional PCR was utilized to amplify the DNA of β-tubulin gene isotype 1

Table 4: PCR sensitivity calculation table

	Microscopy	
	Positive	Negative
PCR		
Positive	a	b
Negative	c	d

*Sensitivity is calculated by (a/a+c×100) and specificity is by (d/b+d×100)

and 18S rRNA genus-specific primers was used in this study (Table 1). It is a mitochondria gene which is conserved and conserved among STHs species.

The β-tubulin gene isotype I and conventional PCR protocol was carried out using 2xPCR Master mix Solution (i-pfu) with sense and antisense primers as mentioned in Table 1 and 2. The initial denaturation was at 94°C for 2 min followed by cycles of denaturation at 94°C for 20 sec. Next step was annealing at 59/55°C (according to the primers) for 10s and elongation at 72°C for 30 sec. Final elongation was set at 72°C for 5 min[18]. The positive control was DNA of *A. lumbricoides* eggs, *T. trichiura eggs* and *S. stercoralis* larvae while parasite-free faeces with for negative controls.

Electrophoresis: The resulting PCR products (DNA that was amplified) were separated on 1% agarose gel based on its size. Prior to gel solidification, the gel was added with ViSafe Red DNA Stain. The image was captured after the electrophoresis with Amersham imager 600. The presence of a band at 158 bp indicates β-tubulin isotype I gene of *A. lumbricoides* while a band at 163 bp indicates *T. trichiura.* The 18S rRNA gene of *S. stercoralis* was detected at 101bp.

Sensitivity calculation: For the sensitivity of PCR technique was compared with microscopic technique[19], the Table 4.

RESULT

PCR sensitivity for *Ascaris lumbricoides*: For *Acaris lumbricoides*, all samples were positive by microscope and only six were positive by PCR technique (Table 5) (Fig. 2).

PCR sensitivity for *Trichuris trichiura*: For *Trichuris trichiura*, all samples were positive by microscope and only two were positive by PCR technique (Table 6) (Fig. 3).

PCR sensitivity for *Strongyloides stercoralis*: For *S. stercoralis*, microscopic observations revealed that all of the stool samples did not contain any *S. stercoralis* larvae or egg. However, the presence of unidentified helminths can be seen in these stool samples which implied that these stool

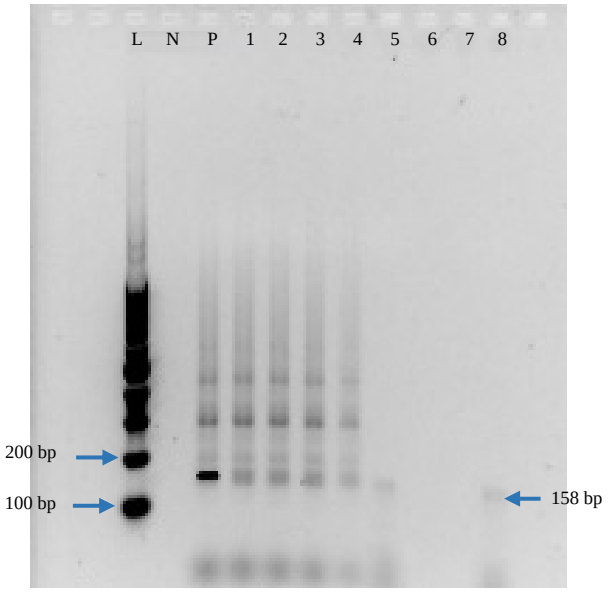

Fig. 2: PCR analysis for *Ascaris lumbricoides*
Lane L: 100 bp of DNA marker, Lane N: Negative control, Lane P: Positive control, Lane 1-8: Stool sample, Lanes 1-5 and 8: Specimens positive for *A. lumbricoides*

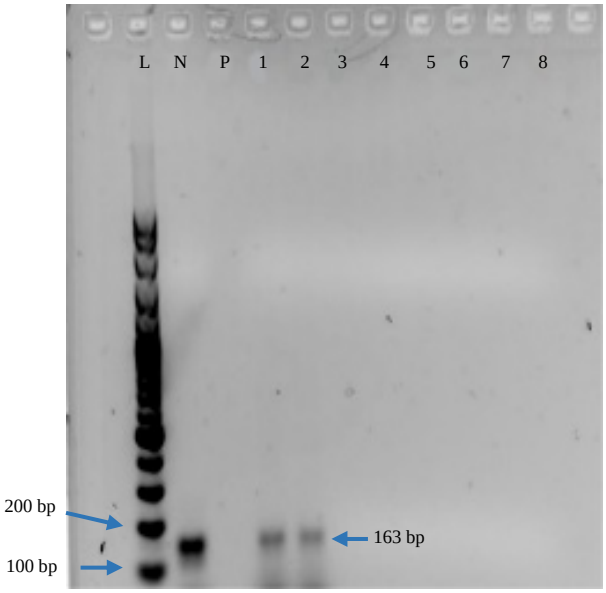

Fig. 3: PCR results for *T. trichiura*
Lane L: 100 bp of DNA marker, Lane N: Negative control, Lane P: Positive control, Lane 1-8: Stool sample, Lanes 1-2: specimens positive for *T. trichiura*

samples did carry other helminths. These microscopic findings indicated that all these stool samples were negative for *S. stercoralis* but positive for other helminths (Fig. 4).

However, there were no DNA amplification of *S. stercoralis* was seen at 101 bp in gel electrophoresis for all samples that

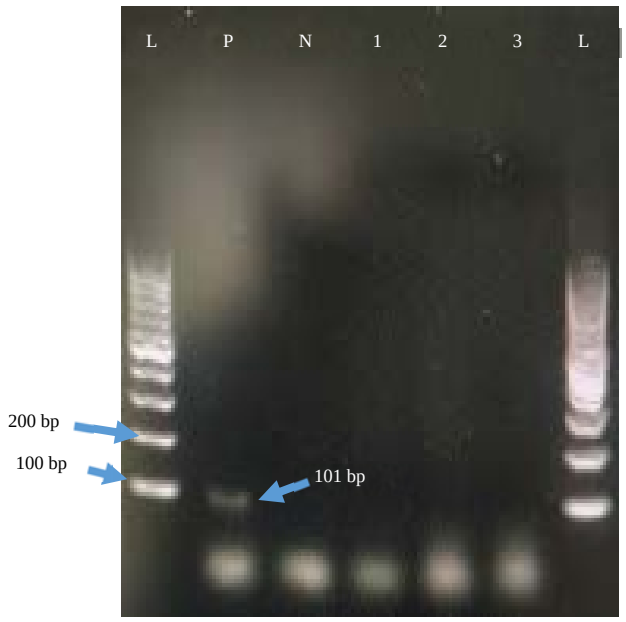

Fig. 4: Gel electrophoresis of PCR results for *S. stercoralis* for 3 stool samples

Table 5: Comparison of microscopy and PCR techniques to detect *A. lumbricoides*

	Microscopy		
	Positive	Negative	Total
PCR			
Positive	6	0	6
Negative	2	0	2
Total	8	0	8

Sensitivity of PCR techniques in detecting *A. lumbricoides* is 75% as compare to microscopy (100%)

Table 6: Comparison of microscopy and PCR techniques to detect *T. trichiura*

	Microscopy		
	Positive	Negative	Total
PCR			
Positive	2	0	2
Negative	6	0	6
Total	8	0	8

For *T. trichiura*, the sensitivity of PCR techniques in detecting is 20% as compare to microscopy (100%)

was tested as shown in Fig. 4. It can be said that all tested samples were negative for *S. stercoralis* by PCR technique.

DISCUSSION

In this study, all tested samples were positive for microscopy technique except for *S. stercoralis* and the same samples were used for molecular testing by conventional PCR. For *A. lumbricoides*, six out of eight samples showed positive amplification via PCR (75%) as compared to microscopy (100%). Meanwhile two out of eight samples were positive (20%) as compared to microscopy (100%) for *T. trichiura*. Quantitative analysis was not included in this preliminary study thus eggs per gram (epg) were not counted and small samples was used. As there is no true gold standard methods, microscopy techniques remain the cornerstone for the detection of STHs infection as they serve sensitive qualitative determination of the infection status, rapid as well as inexpensive[14]. Nonetheless, the morphology of STHs can be distorted during the processing or sampling and isolation of eggs methods that are prone to human error[16]. Additionally, subspecies of STHs are indistinguishable by these techniques beside it has low sensitivity to light infection or following anthelminthic drugs treatment[20,21].

Development of a diagnostic PCR assay to identify intestinal parasite infecting human is sensitive and specific but it also can be challenged by several factors like the physical and chemical nature of the feces itself where the parasite present, the genetic materials of these parasites are usually enclosed in very sturdy cell walls and presence of heme, bilirubin, bile salts, carbohydrates and other components which may serve as PCR inhibitors[22]. Thus, those components of feces can lead to poor recovery of DNA as well as constrain polymerase activity if co-extracted with the target gene and produce false-negative results[23]. Therefore, one of the reasons that cause absence in PCR amplification maybe due to the presence of PCR polymerase inhibitors in stool samples in the washing and flotation analysis step. Therefore, in order to overcome these obstacles, several processing procedures to feces have been adopted from previous studies prior and during the DNA extraction[22].

A few steps of washing and sieving with a combination of wet cheese cloths can enhance the eggs recovery in flotation analysis[24] by removing larger particles that impede eggs detection. Nevertheless, there is a possibility for the eggs to lose if it is still clumped together with the discard materials retained on the sieve. So, there is a need to homogenize or dissociate the eggs from fecal matrix before filtration to reduce those risk[16]. The size of mesh to the size of targeted STHs eggs also needs to be matched carefully to avoid retain the eggs on the sieve. A previous study stated that, use of more than three layers of gauze reduced the concentration index of eggs as many eggs were trapped on the gauze. In contrast, the usage of three layers of gauze with washing increase the concentration index of eggs remarkably[24]. Besides, washing and sieving also assist to concentrate the eggs in a small volume[14]. Flotation technique is also crucial as

it was used to isolate the larvae and eggs of STHs[16] from other particles of different densities by concentrating them at a specific density range with identifiable position in the tube[23].

Gawor *et al.*,[15] stated that *Ascaris* eggs need mechanical damage of their shell or enzymatic lysis such as proteinase K for an extended period to extract DNA efficiently for PCR. Besides, mechanical disruption are superior to enzymatic and chemicals methods as it does not involve addition of reagents that may intrude other subsequent reactions[25]. Some protocols suggest a few egg disruption methods for instance freeze-thaw cycle, heating/boiling or shaking with glass beads[14,16]. Due several unsuccessful trials to lyse the *A. lumbricoides* and *T. trichiura* eggs with proteinase K for overnight, repeated freezing following boiling method was performed as the eggs were proven to be susceptible to lysis and released ample amount of DNA for successful amplification using conventional PCR[13]. However, it could be partial STHs lysis that could have contributed to the absence of PCR products that lead to false-negative results. Additionally, 100% disruptions of egg can also be achieved by using a bead beater system with zirconia/silica or ceramic beads for 1 min, which was not available in laboratories during the time of study[25].

There were some issues using QIAamp DNA Mini Kit (QIA) and it was performed with some modification. Previous studies also agreed revealed that DNA with QIAamp DNA Stool Mini Kit was not very successful. According to Barda *et al.*[26], low performance of DNA Stool Mini Kit was probably due to short lysis period (5 min at 95°C) in DNA stool kits method because of insufficient time to lyse helminths eggs and worms. In addition, DNA Mini Kit showed a significantly higher sensitivity (78% versus 31%) as compared to DNA Stool Mini Kit because it could be used even without a cell lysing instrument. A series of optimization was made for the DNA Mini Kit as suggested by Hawash[22].

The existence of PCR inhibitors can prevent DNA amplification by PCR is another consideration which should be addressed in PCR assays using stool samples[27]. Generally, PCR inhibitors demonstrate its effects by direct contact with DNA or interaction with thermostable DNA polymerases during PCR procedure. Direct attachment of these agents with single or double-stranded DNA can prohibit amplification as well as enable co-purification of the inhibitor and DNA. In addition, inhibitors may also interfere with a DNA polymerase specifically to inhibit the action of the enzyme[28]. These inhibitors including fats, phenolic compounds, cellulose, glycogen and heavy metals may be derived from dietary components and may result in false-negative tests of PCR. The introduction of BSA to the cDNA and PCR processes appeared to be a simple and efficient approach to remove the inhibitory effect of these substances[29].

From the stool samples that were tested during this study, all of the samples were negative for *S. stercoralis* through microscopic examination and were not amplified by conventional PCR assay. Thus, it indicates that these samples may have not contain any *S. stercoralis* in the stools. Absence of PCR products could be due to the presence of PCR inhibitor as well as low DNA yield during isolation. Apart from that, irregular excretion of *S. stercoralis* larvae or the presence of dead larvae may clarify the failure of microscopic examination to identify these stool samples. Furthermore, PCR does not rely on the viability of parasites to detect *S. stercoralis* in the samples[30]. Other than that, the DNA isolation protocol was slightly modified due to lack of lab equipment when conducted this study. According to the manual that has been provided, the samples need to incubate at 95°C in a thermal mixer with shaking at 900 rpm. However, the mixer incubator was used instead of the thermal mixer and the sample was incubated at 65°C under continuous shaking at 200 rpm due to the unavailability of the thermal mixer during this study was conducted, even at 72°C the DNA will be stable not possible 65°C can destroy DNA.

CONCLUSION

In this study, simultaneous detection of three species of STH using a standardize kit protocol combined with boiling and freeze thaw methods for conventional PCR analysis were successfully designed. However, we found this assay had a lower sensitivity compared to the microscopy method. Concisely, additional study needs to standardize conventional PCR protocol using other kits for comparison with microscopy techniques to diagnosing STHs.

SIGNIFICANCE STATEMENT

This study establishes the use of a physical pre-treatment protocol for STH DNA extraction. This method can be future extrapolated using other DNA extraction kits. In fact, this pre-treatment method can be tested on protozoa parasite which has a hardy cyst covering their parasite cell wall.

REFERENCES

1. Dunn, J.C., H.C. Turner, A. Tun and R.M. Anderson, 2016. Epidemiological surveys of and research on, soil-transmitted helminths in Southeast Asia: A systematic review. Parasites Vectors, Vol. 9. 10.1186/s13071-016-1310-2.
2. Cunningham, L., J. Odoom, D. Pratt, L. Boatemaa, N. Asante-Ntim and K. Attiku *et al*., 2018. Expanding molecular diagnostics of helminthiasis: Piloting use of the GPLN platform for surveillance of soil transmitted helminthiasis and schistosomiasis in Ghana. PLoS Negl. Trop. Dis., Vol. 12. 10.1371/journal.pntd.0006129.
3. Silver, Z., S. Kaliappan, P. Samuel, S. Venugopal, G. Kang, R. Sarkar and S. Ajjampur, 2018. Geographical distribution of soil transmitted helminths and the effects of community type in South Asia and South East Asia-A systematic review. PLoS Negl. Trop. Dis., Vol. 12. 10.1371/journal.pntd.0006153.
4. Ngui, R., S. Ishak, C. Chuen, R. Mahmud and Y. Lim, 2011. Prevalence and risk factors of intestinal parasitism in rural and remote West Malaysia. PLoS Negl. Trop. Dis., Vol. 12. 10.1371/journal.pntd.0000974.
5. Cepon-Robins, T., M. Liebert, T. Gildner, S. Urlacher, A. Colehour and J. Snodgrass *et al*., 2014. Soil-transmitted helminth prevalence and infection intensity among geographically and economically distinct shuar communities in the ecuadorian amazon. J. Parasitol., 100: 598-607.
6. Olsen, A., L. van Lieshout, H. Marti, T. Polderman and K. Polman *et al*., 2009. Strongyloidiasis-the most neglected of the neglected tropical diseases? Trans. R. Soc. Trop. Med. Hyg., 103: 967-972.
7. Ahmad, A.F., F. Hadip, R. Ngui, Y.A.L. Lim and R. Mahmud, 2013. Serological and molecular detection of *Strongyloides stercoralis* infection among an orang asli community in Malaysia. Parasitol. Res., 112: 2811-2816.
8. Taniuchi, M., L. Lieshout, R. Haque, S. Sobuz, J. Verweij and W. Petri *et al*., 2011. High throughput multiplex pcr and probe-based detection with luminex beads for seven intestinal parasites. Am. J. Trop. Med. Hyg., 84: 332-337.
9. Rashwan, N., A. Diawara, M. Scott and R. Prichard, 2017. Isothermal diagnostic assays for the detection of soil-transmitted helminths based on the SmartAmp2 method. Parasites Vectors, Vol. 10. 10.1186/s13071-017-2420-1.
10. Lamberton, P.H.L. and P.M. Jourdan, 2015. Human ascariasis: Diagnostics update. Curr. Trop. Med. Rep., 2: 189-200.
11. Khurana, S. and S. Sethi, 2017. Laboratory diagnosis of soil transmitted helminthiasis. Trop. Parasitol., 7: 86-91.
12. Gonçalves, A., R. Abellana, H. Pereira-da-Silva, I. Santos, P. Serra and G. Julião *et al*., 2014. Comparison of the performance of two spontaneous sedimentation techniques for the diagnosis of human intestinal parasites in the absence of a gold standard. Acta Trop., 131: 63-70.
13. Nisha, M., M.R. Su, N. Nadiah and F. Davamani, 2020. Additional egg disruption techniques facilitate better DNA yield for species level identification of *Ascaris lumbricoides* and *Trichuris trichiura*. Res. J. of Parasitol., 15: 31-37.
14. Demeler, J., S. Ramünke, S. Wolken, D. Ianiello, L. Rinaldi and J. Gahutu *et al*., 2013. Discrimination of gastrointestinal nematode eggs from crude fecal egg preparations by inhibitor-resistant conventional and real-time PCR. PLoS ONE, Vol. 8. 10.1371/journal.pone.0061285.
15. Gawor, J., J. Gawor, R. Gromadka, T. Zwijacz-Kozica and F. Zięba, 2017. A modified method for molecular identification of *Baylisascaris transfuga* in European brown bears (Ursus arctos). Parasitol. Res., 116: 3447-3452.
16. Collender, P.A., A.E. Kirby, D.G. Addiss, M.C. Freeman and J.V. Remais, 2015. Methods for quantification of soil-transmitted helminths in environmental media: Current techniques and recent advances. Trends Parasitol., 31: 625-639.
17. Huggins, L., C. Michaels, S. Cruickshank, R. Preziosi and K. Else, 2017. A novel copro-diagnostic molecular method for qualitative detection and identification of parasitic nematodes in amphibians and reptiles. PLoS ONE, Vol. 12. 10.1371/journal.pone.0185151.
18. Rashwan, N., M. Scott and R.Prichard, 2017. Rapid genotyping of β-tubulin polymorphisms in *Trichuris trichiura* and *Ascaris lumbricoides*. PLoS Negl. Trop. Dis., Vol. 11. 10.1371/journal.pntd.0005205.
19. Trevethan, R., 2017. Sensitivity, specificity and predictive values: Foundations, pliabilities and pitfalls in research and practice. Front. Public Health, Vol. 5. 10.3389/fpubh.2017.00307.
20. Sitta, R.B., J.R. Pinho, P.P. Chieffi and R.C.B. Gryschek, 2014. Conventional PCR for molecular diagnosis of human strongyloidiasis. Parasitology, 141: 716-721.
21. Shiraho, E., A. Eric, I. Mwangi, G. Maina, J. Kinuthia and M. Mutuku *et al*., 2016. Development of a loop mediated isothermal amplification for diagnosis of *Ascaris lumbricoidesin* fecal samples. J. Parasitol. Res., Vol. 2016. 10.1155/2016/7376207.
22. Hawash, Y., 2014. DNA extraction from protozoan oocysts/cysts in Feces for diagnostic PCR. Korean Soci. Parasitol. Trop. Med., 52: 263-271.
23. Jacobson, M., B. Norling, A. Gunnarson and A. Aspan, 2009. Flotation-A new method to circumvent PCR inhibitors in the diagnosis of *Lawsonia intracellularis*. Int. J. Microbiol., Vol. 2009. 10.1155/2009/410945.
24. Uga, S., K. Tanaka and N. Iwamoto, 2010. Evaluation and modification of the formalin–ether sedimentation technique. Trop. Biomed., 27: 177-184.
25. Harmon, A.F., D.S. Zarlenga and M.B. Hildreth, 2006. Improved methods for isolating DNA from *Ostertagia ostertagi* eggs in cattle feces. Vet. Parasitol., 135: 297-302.

26. Barda, B., R. Wampfler, S. Sayasone, K. Phongluxa and S. Xayavong *et al*., 2018. Evaluation of two DNA extraction methods for detection of *Strongyloides stercoralis* infection. J. Clin. Microbiol., Vol. 56. 10.1128/jcm.01941-17.
27. Repetto, S.A., C.D.A. Soto, S.I. Cazorla, M.L. Tayeldin and S. Cuello *et al*., 2013. An improved DNA isolation technique for PCR detection of *Strongyloides stercoralis* in stool samples. Acta Trop., 126: 110-114.
28. Schrader, C., A. Schielke, L. Ellerbroek and R. Johne, 2012. PCR inhibitors-occurrence, properties and removal. J. Applied Microbiol., 113: 1014-1026.
29. Oikarinen, S., S. Tauriainen, H. Viskari, O. Simell, M. Knip, S. Virtanen and H. Hyöty, 2009. PCR inhibition in stool samples in relation to age of infants. J. Clin. Virol., 44: 211-214.
30. Verweij, J.J., M. Canales, K. Polman, J. Ziem, E.A.T. Brienen, A.M. Polderman and L. van Lieshout, 2009. Molecular diagnosis of *Strongyloides stercoralis* in faecal samples using real-time PCR. Trans. R. Soci. Trop. Med. Hyg., 103: 342-346.