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Review

# Advantages and limitations of microscopy and molecular detections for diagnosis of soil-transmitted helminths: An overview

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**Summary**

World Health Organization (WHO) reported that over 1.5 billion people are infected by soil-transmitted helminths (STH) worldwide in sub-Saharan Africa, the United States of America, China, and East Asia. Heavy infections and polyparasitism are associated with higher morbidity rates, and the patients are exposed to increased vulnerability to other diseases. Therefore, accurate diagnosis followed by mass treatment for morbidity control is necessary.STH diagnosis commonly involves the microscopic observation of the presence of the STH eggs and larvae in the faecal samples. Furthermore, molecular approaches are increasingly utilised in monitoring and surveillance as they show higher sensitivity. Their capability to differentiate hookworm species is an advantage over the Kato-Katz technique. This review discusses the advantages and limitations of microscopy and various molecular tools used for STH detection.
**Keywords:** soil-transmitted helminths; hookworm; *Ascaris lumbricoides; Trichuris trichiura; Strongyloides stercorali;* microscopic; molecular

## Introduction

Infections caused by soil-transmitted helminths (STH) are often neglected in tropical countries, as declared in the London Declaration of Neglected Tropical Diseases (NTD) 2012. However, STH infection is highly prevalent in under-developed countries and results in chronic illness (Werkman *et al.*, 2018; Basuni *et al.*, 2011). The resulting impacts on the education and economy of the affected nations remain unquantified (Parija *et al.*, 2017). There are four main human-infecting STH species, specifically the roundworm (*Ascaris lumbricoides*), whipworm (*Trichuris trichiura*), and hookworms (*Necator americanus* and *Ancylostoma* sp.) (Ngwese *et al.*, 2020; Werkman *et al.*, 2018). Although the threadworm (*Strongyloides stercoralis*) is not included on the list of NTD by World Health Organization (WHO), its geographical distribution overlaps with other STH. It has high poverty-related morbidity (Ngwese *et al.*, 2020).

WHO reported that over 1.5 billion people are infected by STH worldwide, with their significant occurrence in sub-Saharan Africa, the United States of America, China, and East Asia (WHO, 2020). The infections caused by STH remain endemic in these regions, with the most impoverished sub-Saharan Africa and several regions in South-East Asia suffering a more significant impact (Parija *et al.*, 2017; Pullan *et al.*, 2014). *A. lumbricoides* is the most common STH where it has infected around 1.2 billion people, followed by *T. trichiura*, *A. duodenale* and *N. americanus,* with 740 – 795 million people infected (Hailegebriel *et al.*, 2020). The prevalence of *S. stercoralis* is underestimated globally due to the low sensitivity of diagnostic tests and the inefficiency of case reporting in high-incidence countries (Mora & Meseeha, 2022). *S. stercoralis* is

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widespread globally except in the far north and far south regions, where it is commonly found in warm and moist areas with poor sanitary conditions (Mora & Meseeha, 2022).
Human infection by *A. lumbricoides* results in the common tropical infection ascariasis (Shiraho *et al.*, 2016). Adult *A. lumbricoides* causes upper gastrointestinal bleeding, small bowel obstruction, volvulus and intussusception, peritonitis, and gastric ascariasis with possible perforation (Jourdan *et al.*, 2018). A *T. trichiura* infection is commonly asymptomatic as the clinical disease depends on parasite burden (Viswanath *et al.*, 2022). However, it can be symptomatic, accompanied by abdominal pain, painful passage of stools and abdominal discomfort. Trichuris Dysentery Syndrome signs such as anaemia could be present in severe cases (Viswanath *et al.*, 2022; Jourdan *et al.*, 2018). Individuals infected with *S. stercoralis* may experience irritation at the site on the skin where the larvae penetrated, followed by irregular localised oedema or urticaria (Nutman, 2017). *S. stercoralis* is associated with dermatological and gastrointestinal morbidity in children (WHO, 2020). On the other hand, hookworms rarely cause severe anaemia or significant protein loss in the host. Thus, the annual death is lower compared to other STH infections. However, hookworm infections impose long-term effects such as malnutrition and delayed cognitive development, particularly in children (Bethony *et al.*, 2006).
WHO recommends annual and biannual regional mass drug administration (MDA) program in endemic populations by delivering 400 mg single dose albendazole or 500 mg mebendazole (Stracke *et al.*, 2019). The MDA is an effort to deworm high-risk population groups such as pre-school-aged children (PSAC), school-aged children (SAC), and women of reproductive age (WRA) (Manuel *et al.*, 2021). Since morbidity is more frequent in children than adults, adults are usually excluded from MDA programs except for hookworm infection in pregnant women (Werkman *et al.*, 2018). The necessity of deworming in an area or country is determined by population-level parasitological studies, which evaluate the prevalence and severity of infection (Manuel *et al.*, 2021).
STH detection is commonly based on the microscopic diagnosis of the helminth eggs or larvae in human stool and occasional shedding of the eggs or larvae of the parasites (Glinz *et al.*, 2010). However, the availability of well-trained professionals is less in low prevalence areas, often misdiagnosed (Khurana & Sethi, 2017; O'Connell & Nutman, 2016). The Kato-Katz thick stool smear is still recommended in the WHO 2030 roadmap as the standard diagnostic to detect and quantify the intensity of STH infections. However, the Kato-Katz technique is not recommended to diagnose Strongyloidiasis infections due to its lower sensitivity for STH with low intensity of infection, despite its ideal application for diagnosing moderate and high-intensity infection (M & HI) (Stuyver & Levecke, 2021). Besides, the sample stirring step decreases faecal egg count variation for hookworm and *T. trichiura* even though *A. lumbricoides* diagnosis is not affected (Bosch *et al.*, 2021). Although the traditional coprological methods are reliable and widely used to detect parasite larvae, the need for enhanced sensitivity is growing as a measure for STH control (Papaiakovou *et al.*, 2019). Therefore, it is crucial to develop new diagnostics to monitor, control and eliminate STH (Knopp *et al.*, 2014)
With the increasing emphasis on diagnostic accuracy, molecular methods are gaining attention as alternatives to overcome the drawbacks of microscopy-based techniques (Azzopardi *et al.*, 2021). The advantages of molecular methods are they provide higher sensitivity and specificity than microscopy and are constantly improving due to new technologies in genomics and bioinformatics(Papaiakovou *et al.*, 2021).On the other hand, FECPAK$^{G2}$ is a newly developed quantitative method for the microscopic quantification and detection of STH eggs in a human stool(Ayana *et al.*, 2018). FECPAK$^{G2}$ involves a special microscope equipped with an electronic camera to capture the image of the sample (Boelow *et al.*, 2022). The image is stored and shared through cloud storage to provide a reference for settings with the limited resource (Moser *et al.*, 2018). Therefore, based on the current research in this area, this review discusses the advantages and limitations of the various molecular tools used for STH detection compared to the microscopy-based methods.

## Methodology

For this review, we used PubMed, Google Scholar, Science Direct, ResearchGate, World Organization Health (WHO) website web to do a web searching using the following keywords: "Diagnosis soil-transmitted helminths" OR "*S. stercoralis*" OR "*T. trichiura*" OR "hookworm, OR "*A. lumbricoides*" OR "*A. duodenale*" OR "*N. americanus*" OR "nematodes" OR "roundworm" OR "whipworm" OR "helminth" OR "soil-transmitted helminths" OR "worm burden" OR "Kato-Katz" OR "McMaster" OR " FLOTAC" OR "Real-Time PCR" OR "PCR" OR "LAMP". Our searching covered articles published between 2000 and 2022.

## Advantages and limitations: Microscopy-based Techniques

Microscopy-based techniques are simple and low-cost, but their sensitivity is affected by several factors, including intermittent excretion of parasite ova, low infection intensity that limits the detection, inappropriate transportation or sample storage (Khurana *et al.*, 2021; Papaiakovou *et al.*, 2019). Table 1 summarises several microscopy-based diagnostic methods for STH diagnosis.
The direct wet mount examination of moist stool samples has the advantage of detecting the motile trophozoite stage of the protozoan species (Demeke *et al.*, 2021). Direct wet mount microscopy is widely used in Africa, particularly in Ethiopia, to diagnose parasitic intestinal infections (Demeke *et al.*, 2021; Mengist *et al.*, 2018). Meanwhile, the formol–ether concentration (FEC) method helps detect STH eggs in a large sample of fixed stool (Speich *et al.*, 2014). Nevertheless, it shows substandard accuracy in detecting threadworm larvae (Glinz *et al.*, 2010), and hookworm eggs were

Table 1. Microscopy-based diagnostic methods for soil-transmitted helminths diagnosis.

| Microscopy-based techniques | Procedure | Outcome | Sensitivity | Negative Predictive Value | Advantages | Limitation | References |
|---|---|---|---|---|---|---|---|
| Direct wet mount Microscopy | Preparation of stool sample with one drop of saline/iodine on a microscopic slide with a cover glass. | Egg detection | Sensitivity: *A. lumbricoides* 83.3% and Hookworm 85.7%<br><br>Sensitivity: Hookworm: 37.9 %, *A. lumbricoides* 52% and *T. trichiura* 12.5% | Hookworm 97.5% and *A. lumbricoides* 98.8% | Low cost, easy and able to detect motile trophozoite | Low sensitivity | (Demeke *et al*., 2021; Dana *et al*., 2020; Else *et al*., 2020; Mengist *et al*., 2018 ; Nikolay *et al*., 2014; Endris *et al*., 2012) |
| Formol-ether concentration (FEC) | The stool sample is added to 7 mL of 10% formalin and the suspension is filtered through a sieve into a 15 mL conical centrifuge tube. Then, 4 mL diethyl ether is added to the formalin solution and centrifuged at 300 rpm for 1 min. The supernatant is discarded and the smear is prepared using a slide from the sediment. The sediment is screened under a microscope. | Egg detection | Sensitivity: *A. lumbricoides* 32.5% hookworm 64.2% and *T. trichiura* 75%<br><br>Sensitivity: *A. lumbricoides* 81.4% hookworm 72.4% and *T. trichiura* 57.8% | *A. lumbricoides* 94.7%, hookworm 84.5% and *T. trichiura* 75%<br><br>*A. lumbricoides* 93%, hookworm 97% and *T. trichiura* 91.5% | Low cost Essential for helminths ova detection | Need for centrifugation and does not detect unembryonated eggs of *Ascaris* spp. | (Hailu *et al*., 2022; Demeke *et al*., 2021; Else *et al*., 2020; Fenta *et al*., 2020; Sam *et al*., 2018; Endris *et al*., 2012 ) |
| Kato-Katz | The stool sample is pressed through a mesh screen to remove particles and an amount of sieved stool sample is placed into the hole of the template on a slide. Then, the template is removed and the remaining sample is covered with cellophane which is previously immersed with Glycerol-malachite green. The sample is screened under a microscope after 30 min and the eggs are counted. | Egg detection and egg quantification | Sensitivity: *A. lumbricoides* 93.1%, hookworm 69% and *T. trichiura* 90.6%<br><br>Sensitivity: *A. lumbricoides* 50%, hookworm 55.7% and *T. trichiura* 75%<br><br>Sensitivity: *A. lumbricoides* 73%, hookworm 75.3% and *T. trichiura* 90.9%<br><br>Sensitivity: *A. lumbricoides* 89.8%, hookworm 89.1% and *T. trichiura* 96.1% | *A. lumbricoides* 97.3%, hookworm 97.3% and *T. trichiura* 98%<br><br>*A. lumbricoides* 96%, hookworm 81.5% and *T. trichiura* 99.8% | WHO recommended gold standard, low-cost, possible to determine the burden of infection | Low sensitivity, fails to detect infections of low intensity | ( Khurana *et al*., 2021; Fenta *et al*., 2020; Cools *et al*., 2019; Moser *et al*., 2018; Endris *et al*., 2012) |

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| McMaster | A known amount of stool is mixed with a saturated salt solution. The top layer of the solution is added to the reading chamber and the EPG count can easily be derived by multiplying the number of eggs under the marked areas by a simple conversion factor | Egg quantification | Sensitivity: *A. lumbricoides* 74.3%, hookworm 67.6% and *T. trichiura* 94.6%<br>Sensitivity: *A. lumbricoides* 74.3% , hookworm 92.3% and *T. trichiura* 94.9% | *A. lumbricoides* 82.4%, hookworm 62% and *T. trichiura* 47.8% | Inexpensive, easy, has been extensively used in human studies for estimation of anthelmintic cure rates.<br>Highly sensitive for low-intensity soil-transmitted helminth infectionS | Need for a special counting chamber | (Khurana *et al.*, 2021; Manuel *et al.*, 2021; Else *et al.*, 2020; Albonico *et al.*, 2013; Albonico *et al.*, 2012) |
| FLOTAC | The FLOTAC apparatus is a cylindrical-shaped device made of polycarbonate amorphous thermoplastic with two flotation chambers. An amount of stool sample is homogenized and filtered with water or saline. The filtrate is mixed with a floatation solution and is added to the chambers. The chambers are centrifuged and examined under a microscope. | Egg quantification | Sensitivity: *A. lumbricoides* 81.9%, hookworm 80.% and *T. trichiura* 96.8%<br>Sensitivity: *A. lumbricoides* 79.7%, hookworm 92.4.% and *T. trichiura* 91% | *A. lumbricoides* 86.9%, hookworm 73.4% and *T. trichiura* 61.1% | Detection of different STHs simultaneously especially for low-intensity infections. | It is complex, a specific device is needed and requires centrifugation with two different rotors. | (Khurana *et al.*, 2021; Manuel *et al.*, 2021; Else *et al.*,2020; Knopp *et al.*,2014;Nikolay *et al.*, 2014; Albonico *et al.*, 2013; Cringoli *et al.*, 2010 ) |
| Mini-FLOTAC | A weighed stool sample is homogenized with 5% formalin, filtered, and the filtrate is added to the flotation solution. The suspension is loaded into the two flotation chambers. Screened under a microscope after 5−10 min. | Egg quantification | Sensitivity: *A. lumbricoides* 42.1%, hookworm 70.8.% and *T. trichiura* 85.6%<br>Sensitivity: *A. lumbricoides* 75.5%, hookworm 79.2.% and *T. trichiura* 76.2% | | Detection of different STHs simultaneously | It is complex and a specific device is needed. Less sensitive for the diagnosis of *A. lumbricoides* | ( Khurana *et al.*, 2021; Manuel *et al.*, 2021; Else *et al.*, 2020; Cools *et al.*, 2019; Barda *et al.*, 2013; Nikolay *et al.*, 2014) |

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| Baermann Technique | A 10 g of stools is place over few layers of gauze inserted into a cup filled with tap water. The apparatus is expose to artificial light directed at the funnel's bottom. Then 50 ml of the liquid is collected from the bottom of funnel in a plastic tube after 2 hours and centrifuged at 500 *g* for 2 minutes. A water suction pump use to drain the supernatant. | Larvae detection | Sensitivity: *S. stercoralis* 16%<br><br>Sensitivity: *S. stercoralis 20.7*%<br><br>Sensitivity: 28.3% and specificity of 75.2% | | Highest sensitivities for *S. stercorali*s and detected larvae can be identified more easily<br>Nearly 4 times higher sensitivity than FEC | Time consuming, large quantity of stool required, fresh sample required. | ( Khurana *et al*., 2021;Hailegebriel *et al*., 2017; Knopp *et al*., 2014; Carvaho *et al.,* 2012; Machicado*et al.,* 2012; Knopp *et al.,* 2008) |
| FECPAK$^{G2}$ | The FECPAK$^{G2}$ platform, which includes a cassette, focuses helminth eggs into a single microscopic field of vision, which is captured and saved on a computer, allowing the eggs to be counted. | Egg quantification | Sensitivity: *A. lumbricoides* 75.6%, hookworm 71.5% , and *T. trichiura* 65.8% | | Detection of different STH simultaneously, simple procedure with a result within an hour | Internet connection is required | (Manuel *et al*., 2021; Else *et al*., 2020;Cools *et al*., 2019; Moser *et al*., 2018) |

easily damaged while processing the samples because the eggshell is very thin and fragile (Panggabean *et al.*, 2017).

The Kato-Katz technique is the most widely used to detect STH infection (Bosch *et al.*, 2021), and it is recommended by WHO for surveillance and epidemiological field surveys of schistosome and STH infections due to its relative simplicity, pace, and low cost (Habtamu *et al.*, 2011). Kato-Katz procedure is modifiable (Khurana *et al.*, 2021), whereby the parasitic load is reported as eggs per gram of stool (EPG) (Azzopardi *et al.*, 2021). The Kato-Katz thick smear is commonly used to detect Ascaris, Trichuris, and the hookworms in which helminth eggs are identified and enumerated by microscopy (Azzopardi *et al.*, 2021). The sensitivity of the Kato-Katz method varies from 65.2 – 96.9 %, with a high specificity of 93.8 – 99.4 % (Azzopardi *et al.*, 2021; Nikolay *et al.*, 2014).

The McMaster (MM) method is another microscopy-based technique that requires a special counting chamber. McMaster is the standard coprological method. It is widely used to assess STH in veterinary parasitology and estimate anthelmintics' cure rates in humans. Its ease of use in estimating faecal egg counts and suitability for unestablished laboratories make it a good choice for public health monitoring of human STH (Periago *et al.*, 2015). On the other hand MM approach seems more suitable for standardisation due to its efficient multiplication factor and ability to detect all STH organisms simultaneously (Levecke *et al.*, 2011).

The FLOTAC technique was first developed for veterinary parasitology before being applied for human parasitology (Cringoli *et al.*, 2010; Utzinger *et al.*, 2008). This technique has recently been proven highly responsive to the Kato-Katz approach for diagnosing hookworm, roundworm, and whipworm (Glinz *et al.*, 2010). Since the FLOTAC technique may analyse stool load of 1 g or more, high multiplication factors are not required to determine faecal EPG (Tello *et al.*, 2012; Glinz *et al.*, 2010). In addition, the Mini-FLOTAC technique is a sensitive and straightforward method for determining the severity of helminth infections (Barda *et al.*, 2013). The Mini-FLOTAC procedure does not require any centrifugation steps or costly facilities. Both fresh and fixed stool samples can be used for this procedure, and the whole process only takes 10 – 12 min (Barda *et al.*, 2013). The faecal egg count and the number of helminth eggs are counted per species and multiplied by 10 (Dana *et al.*, 2020).

The Baermann Technique (BM) is another microscopy-based technique that requires a glass funnel that used for the detection of nematode larvae in faecal samples and is based on the thermotropism and hydrotropism of the larvae (Carvaho *et al.*, 2012). The Baermann Technique (BM) is a diagnostic method for detecting *S. stercoralis* (Knopp *et al.*, 2008). Larvae detection and first-stage *S. stercoralis* larvae (L1) confirmation is carried out under a microscope at 100x magnification and 400x magnification respectively. Although the BM technique has low positivity rates of parasite detection, it shows moderate sensitivity in detecting *S. stercoralis* infection with a sensitivity of 28.3 % and specificity of 75.2 % (Hailegebriel *et al.*, 2017; Knopp *et al.*, 2014; Carvaho *et al.*, 2012).

FECPAK$^{G2}$ is developed to produce digital images of helminth eggs that have been concentrated into one microscopic field of view, and the images can be backed up online (Ayana *et al.*, 2018). FECPAK$^{G2}$ achieved a detection sensitivity of 75.6 % for *A. lumbricoides*, 71.5 % for hookworm and 65.8 % for *T. trichiura* from human stools in a randomised controlled trial with increased sensitivity for moderate infection intensities (Moser *et al.*, 2018). However, faecal FEC given by Mini-FLOTAC and FECPAK$^{G2}$ differed markedly and led to a false classification for the low load intensity samples, thus highlighting the need for method-specific infection intensity thresholds (Cools *et al.*, 2019).

**Principles and advantages of molecular-based techniques**

The molecular approaches include conventional polymerase chain reaction (PCR), real-time PCR, digital PCR, loop-mediated isothermal amplification assay (LAMP) and, more recently, cell-free DNA detection and quantitative paper-based DNA reader. Various molecular diagnostic tests for STH diagnosis are summarised in Table 2.

Molecular-based methods can detect and identify intestinal parasites in faecal samples (Rogers *et al.*, 2021) from appropriate DNA targets (Verweij *et al.*, 2001). Advances in the bioinformatics sequencing of nematode genomes with the availability of nematode sequence data have enabled the development of assays which target species-specific genomic regions (Khurana *et al.*, 2021; Grant *et al.*, 2019). The DNA sequences that are commonly used as targets for the detection of STH are ribosomal internal transcribed spacer (ITS) sequences, 18S or ribosomal sub-unit sequences, mitochondrial genes such as cytochrome oxidase I (COI), and repetitive sequences (Manuel *et al.*, 2021; Pilotte *et al.*, 2016). The first and second internal-transcribed spacer (ITS-1 and ITS-2, respectively) of ribosomal DNA (rDNA) provide genetic markers for the specific identification of parasites, especially *A. duodenale* and *N. americanus* (Gruijter *et al.*, 2005). Ribosomal sequences are chosen as diagnostic targets since they are frequently found in nucleated organisms as easily identified moderate copy number tandem repeats (Pilotte *et al.*, 2016). Mitochondrial genes, particularly cytochrome oxidase-I, are also used as DNA targets due to multiple copies of mitochondrial targets in most eukaryotic cells (Manuel *et al.*, 2021; Pilotte *et al.*, 2016). Non-coding repetitive sequences that are discovered in nearly all eukaryotes could also be used as targets for next-generation sequencing (NGS) owing to their high sensitivity and species-specific STH identification capability (Pilotte *et al.*, 2016). Table 3 shows gene targets used in molecular diagnosis for STH detection.

**Stool Sample Storage and Preservation**

The stool is currently the only validated sample for STH detection due to the biology of the STH infection (Hawkins *et al.*, 2020). Therefore, stool sample storage and DNA extraction are crucial

Table 2. Molecular-based diagnostic methods for soil-transmitted helminths diagnosis.

| Molecular based techniques | | Target | STH | Sensitivity and specificity / Rate of Detection | Advantages | Disadvantages | References |
|---|---|---|---|---|---|---|---|
| Conventional PCR | Singleplex | Cytochrome Oxidase | *A. duodenale and* *N. americanus* | | 1. Rapid, technically straightforward, and highly sensitive<br>2. Able to identify individuals with mixed infections<br>3. Workable for fresh or preserved fecal samples<br>4. Low cost | Results must be visualized through gel electrophoresis | (Fleitas *et al*., 2021; George *et al*., 2015; Ngui *et al*., 2012; Nilforoush *et al*., 2007; J J Verweij *et al*., 2001; Zhan *et al*., 2001) |
| | Semi nested PCR | ITS-2, 28S | *N. americanus* | Sensitivity: 94% Specificity:100% | | | |
| | Nested PCR | ITS-1 | *S. stercoralis* | | | | |
| | Semi-nested PCR-RFLP | ITS-1, ITS 2, 5.8s regions | *A. duodenale and* *N. americanus* | | | | |
| | Multiplex PCR | ITS-1, 18S | *A. lumbricoides* and *N. americanus* *S. stercoralis* | Sensitivity: *S. stercoralis* 97.4% and hookworm 90.3% | | | |
| Real-Time PCR | Singleplex | 18S, ITS-2 | *N. americanus,* *A. duodenale,* *S. stercoralis* | Sensitivity: *S. stercoralis* 88.9% and hookworm 78.9% | 1. Screen multiple samples simultaneously<br>2. Higher sensitivity for all STH<br>3. Fully automated and gel electrophoresis is not required<br>4. Provides quantified results | 1. High usage of expensive consumables<br>2. Not feasible in resource-poor settings<br>3. A high-tech laboratory is required<br>4. Achieving maximized qPCR efficiency is challenging<br>5. Inhibition may occur<br>6. Triplication is required to ensure accuracy | (Othman *et al*., 2020; Benjamin-chung *et al*., 2020; Stracke *et al*., 2019; Cunningham *et al*., 2018; Schaer *et al*., 2013; Wang *et al*., 2012; Ngui *et al*., 2012; Taniuchi *et al*., 2011) |
| | Multiplex | ITS-1, ITS-2, 18S | *A. lumbricoides,* *S. stercoralis,* *A. duodenale* and *N. americanus* | | | | |
| | Multi parallel | Repetitive sequence | *A. lumbricoide,T. trichiura,* *A. duodenale and N. americanus* | Sensitivity: *A. lumbricoides* 79%, hookworm 93%, and *T. trichiura* 90% | | | |
| | PCR-Luminex | ITS-1,2 | *N. americanus,* *A. lumbricoide*s, *A. duodenale, and* *S. stercoralis* | | | | |

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| | Multiplex-tandem PCR-qPCR | *β*-tubulin | *A. lumbricoides, T. trichiura,*<br>*A. duodenale,* and *N. americanus* | Rate of detection:<br>MT-PCR ranged from 90.64% (*A. lumbricoides*s) to 100.00% (*T. trichiura*). | | | |
| | SYBR Green quantification | ITS-2 | *N. americanus* | Rate of detection:<br>22.69% | | | |
| | High Resolution Melting (HRM) | 18S, ITS-1,2 | *A. lumbricoides, T. trichiura, A. duodenale*, and *N. americanus* | Sensitivity: 80 % | | | |
| | | ITS-2 | *N. americanus, Ancylostoma.* spp. | Sensitivity: 100% | | | |
| dPCR | | ITS-1 | *A. lumbricoides* | Rate of detection:<br>20 eggs in 10 L of reclaimed water | 1. Rapid<br>2. Precise<br>3. Sensitive<br>4. Useful in the detection of drug-resistant or high-pathogenicity subpopulations<br>5. Able to detect low amounts of *A. lumbricoides* eggs | The machines and reagents are costly | (Stuyver L.J *et al.*, 2021; Soto *et al.*, 2017) |
| LAMP | SmartAmp2 | *β*-tubulin | *A. lumbricoides, T. trichiura, N. americanus* | | 1. Great sensitivity and specificity<br>2. Low cost<br>3. Simple procedure | A precise and straightforward DNA extraction process is required to avoid the polyphenols found in faecal samples that inhibits DNA polymerase. | (Ngari *et al.*, 2020; Rashwan *et al.*, 2017; Shiraho *et al.*, 2016) |
| | LAMP | ITS-1 | *A. lumbricoides* | Sensitivity: 96.3%<br>Specificity: 61.5% | | | |
| | | ITS-2 | | | | | |

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| quantitative paper-based DNA reader (qPDR) | Singleplex Mini-PCR | *β*-tubulin | *T. trichiura,* | | 1. Low cost<br>2. Simple procedure<br>3. Faster | 1. Required validation<br>2. Need to develop portable stool DNA extraction | (Manuel *et al.*, 2021; Wang *et al.*, 2018) |
| Cell-free DNA | | *18S rRNA, Cox1* | *S. stercoralis* | | 1. Low cost<br>2. Simple procedure<br>3. Faster | Viability and different clearing times of STH eggs because eggs tend to be damaged and are lysed rapidly | (Manuel *et al.*, 2021; Gorgani-Firouzjaee *et al.*, 2018; Weerakoon *et al.*,2016) |

in any molecular assay to assure accuracy. Papaiakovou and co-workers (2018) compared the standard sample storage technique at 20 °C used in most laboratories with different sample preservation techniques. The methods include silica bead two-step desiccation, FTA cards, 5 % potassium dichromate, RNAlater, ethanol, Paxgene, and Formalternate. The comparison was conducted at 4 °C or 32 °C coupled with four collection times of 0 day, 1 day, 7 days and 60 days. In contrast, the control is kept at −20 °C without any preservatives with the same collection time. Stool samples spiked with *N. americanus* ova were used in this investigation. All preservation techniques were stable at 4 °C. Still, high stability was observed only for silica bead two-step desiccation, 5 % potassium dichromate, and FTA card-based preservation conducted at 32 °C, with no significant change in DNA concentration (Manuel *et al.*, 2021; Papaiakovou *et al.*, 2018).

Ayana and co-workers (2019) found that higher concentrations of *A. lumbricoides*, *T. trichiura*, and *N. americanus* DNA can be extracted if the stool samples were preserved with ethanol and RNA than those maintained with potassium dichromate, with no significant changes in sensitivity. In addition, Azzopardi and colleagues (2021) developed a scalable system to wash stool preserved in potassium dichromate. The washed stools could be stored at −30 °C for up to 15 weeks without compromising yield before DNA isolation. Briefly, 7.5 mL of the preserved faeces were poured into a 50 mL tube and centrifuged at 700 x *g* for 3 min to prevent worm egg lysis. The stools were rinsed with 50 mL of phosphate-buffered saline (PBS) and recentrifuged. The material was transferred to a 15 mL tube after a final 15 mL PBS wash followed by centrifugation. The supernatant was removed, and the washed stool pellets were stored at 4 °C for 4 weeks until the DNA extraction.

**DNA Extraction**

The diagnostic performance of DNA-based tests must be evaluated to warrant the quality of diagnostic testing (Cools *et al.*, 2020; Hawkins *et al.*, 2020). The method used for DNA extraction is crucial as it affects the outcome of the assessed tests. Thus, appropriate DNA extraction methodologies are required to increase the sensitivity and accuracy of detection (Sharifdini *et al.*, 2015). Advancement in DNA extraction procedures for STH ova is progressing to develop processes that homogenise faecal samples and break up the outer layer of the egg to achieve high DNA yields (Manuel *et al.*, 2021). Most laboratories prefer commercial kits because of their standardised procedures and relative convenience. However, commercially available kits such as Powersoil DNA Isolation Kit (MO Bio, Carlsbad, CA, USA), FastDNA Spin Kit for Soil (MP Biomedicals, Irvine, CA, USA), QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA, USA) require pre-processing steps such as bead beating and/or lysis which has an important impact on kit sensitivity and DNA recovery (Manuel *et al.*, 2021; Ayana *et al.*, 2019).

**Conventional PCR**

Polymerase chain reaction (PCR)-based methods possess high sensitivity as parasite detection is possible with low DNA concentrations (Manuel *et al.*, 2021; Verweij *et al.*, 2001). A recent study by Fleitas *et al.*, (2021) reported successful detection of *S. stercoralis* and hookworm in stool samples with 2 – 20 pg genomic DNA. The sensitivity for *S. stercoralis* and hookworms was 97.4 % and 90.3 %, while the specificity was 100 % and 87.6 %, respectively (Fleitas *et al.*, 2021). Conventional PCR is also used with other downstream techniques, such as restriction fragment length polymorphism (RFLP), to identify different species of STH (George *et al.*, 2016; Manuel *et al.*, 2021). Based on the work conducted by George and colleagues (2015), a semi-nested PCR-RFLP assay of specific amplification of the internal transcribed spacer gene (ITS) 1, 2, and 5.8s was demonstrated to detect roundworm *A. duodenale*, and animal hookworm *A. ceylanicum* in children from a tribal community in Tamil Nadu, India. On the other hand, Ngui and co-workers (2012) used nested PCR to differentiate between *N. americanus* and *Ancylostoma* spp. in Malaysia with a specific gene target of ITS-2 and 28S ribosomal RNA. In Thailand, Phuphisut and colleagues (2014) employed triplex PCR to detect STH simultaneously in the same reaction, thereby reducing cost. Their study reported that the sensitivity for *A. lumbricoides*, *N. americanus*, and *T. trichiura* detection was 87 %, with a specificity of 83 % where a single copy of DNA was detectable. Nonetheless, agarose gel electrophoresis is required to visualise the results of PCR. The process is time-consuming compared to other molecular diagnostics, contributes to higher costs, and has a potential for contamination (Manuel *et al.*, 2021; Gordon *et al.*, 2011).

**Real-time PCR**

Quantitative real-time PCR (qPCR) or real-time PCR is suitable for detecting DNA targets in stools, even in low-prevalence settings (Papaiakovou *et al.*, 2019). The dyes can be either nonspecific dyes, such as fluorescent DNA-binding dyes (e.g., SYBR Green I) or strand-specific probes (e.g., Taqman or Molecular Beacons) (Fraga, 2008). The detection and monitoring of hookworm *N. americanus* infections can be performed using SYBR Green qPCR, where the ITS-2 sequence of *N. americanus* is amplified for detection (Wang *et al.*, 2012). Besides, probe-based assays such as TaqMan allow multiplexing and multi-parallel detection of multiple helminths from a single stool sample (Benjamin-chung *et al.*, 2020). The higher sensitivity of real-time multiplex PCR compared to that of the Kato-Katz technique is associated with the direct detection of STH DNA in minimal quantities where DNA is less likely to degrade than STH ova (Aung *et al.*, 2022; Benjamin-chung *et al.*, 2020; Llewellyn *et al.*, 2016). It can detect more low-intensity infections compared to the Kato-Katz technique with a limit of detection (LOD) of 10 copies (Basuni *et al.*, 2011; Keller *et al.*, 2020). A recent study conducted by Zendejas-Heredia *et al.*, 2021 re-

Table 3. Gene targets used for molecular diagnosis of soil-transmitted helminths infections.

| STH | Target gene | References |
|---|---|---|
| *Strongyloides stercoralis* | Cytochrome oxidase I<br>Repetitive sequence<br>18S<br>ITS-1<br>ITS-1, 5.8s, ITS-2 | (Basuni *et al.*, 2011; Moghaddassani *et al.*, 2011; Verweij *et al.*, 2009; Nilforoush *et al.*, 2007) |
| *Ascaris lumbricoides* | Repetitive sequence<br>ITS-1<br>ITS-1, ITS-2, 5.8 s | (Pilotte *et al.*, 2016; George *et al.*, 2016; Mejia *et al.*, 2013) |
| *Trichuris trichiura* | Repetitive sequence<br>ITS-1, ITS-2, 5.8 s<br>ITS-1 | (George *et al.*, 2016; Pilotte *et al.*, 2016; Mejia *et al.*, 2013) |
| *Ancylostoma* spp. | Repetitive sequence<br>ITS-1, ITS-2, 5.8 s<br>ITS-2<br>Cytochrome oxidase I | (Pilotte *et al.*, 2016; George *et al.*, 2015; Mejia *et al.*, 2013; Zhan *et al.*, 2001) |
| *Necator americanus* | Repetitive sequence<br>ITS-1, ITS-2, 5.8 s<br>ITS-2<br>Cytochrome oxidase I | (Pilotte *et al.*, 2016; George *et al.*, 2015; Basuni *et al.*, 2011; Zhan *et al.*, 2001) |

vealed that only qPCR was able to classify low-moderate infection cut-offs accurately and able to detect very low infection intensities. A summary of representative studies evaluating the performance of real-time PCR and other methods is provided in table 4.

Real-time PCR can be used in combination with other techniques for better detection. For example, PCR-Luminex, high-resolution melt curve analysis, and multiplexed-tandem. PCR-Luminex is high throughput multiplex PCR combined bead assay involving the binding of PCR products to carboxylated Luminex beads with biotinylated primers and amine-modified probes as an alternative to real-time PCR (Taniuchi *et al.*, 2011). The advantages of PCR-Luminex are higher sensitivity in detection than microscopy, shorter time for technical handling due to the exclusion of several testing modalities such as larvae ova and cysts concentration step, specific staining, or immunoassays, and provide the capacity to perform their tests in-house. The 83 – 100 % sensitivity and specificity were reported based on 319 clinical specimens (Taniuchi *et al.*, 2011). Next, real-time PCR coupled with high-resolution melt curve (HRM) analysis is used to detect different hookworm species, whereby this assay can detect as low as 0.01 ng/mL DNA (Ngui *et al.*, 2012). Furthermore, multiplexed-tandem qPCR (MT-PCR) is a rapid and semi-automated tool that is more user-friendly than conventional multiplex qPCR in identifying and quantifying STH eggs in samples. It displays higher sensitivity than faecal microscopy (Stracke *et al.*, 2019).

**Digital PCR**

Digital PCR (dPCR) is closely related to qPCR as it involves the same amplification reagents. A fluorescent readout indicates adequate amplification of target molecules due to using intercalating agents or hydrolysis-based probe reporters (Salipante & Jerome, 2020). Thus, digital PCR is advantageous over real-time PCR with good repeatability and reproducibility due to the PCR-inhibitory substances which lower the risk of inhibition (Sreejith *et al.*, 2018). Soto and co-workers (2017) demonstrated high sensitivity of dPCR whereby the DNA concentrations of 5 *A. lumbricoides* eggs in reclaimed water equivalents were detected (Soto *et al.*, 2017). Droplet dPCR (ddPCR) is a form of dPCR based on oil-water emulsion technology for partitioning the reaction mixture, which is a potential surveillance tool for detecting STH in environmental samples in endemic areas (Manuel *et al.*, 2021). Despite the diagnostical merits, the implementation of dPCR is challenged by the high cost required to establish the setups.

**Loop-mediated Amplification Assays**

Loop-Mediated Amplification (LAMP) Assay is a one-step DNA amplification method with high specificity and sensitivity where a target sequence is amplified under isothermal conditions (Rashwan *et al.*, 2017). LAMP assay was developed by Notomi *et al*. (2000) with the principle of auto-strand displacement DNA synthesis (Manuel *et al.*, 2021). The assay only requires a water bath or thermal block to provide isothermal conditions (Manuel *et al.*, 2021). LAMP reaction requires no specific reagents and can be performed in any general molecular biology facility (Notomi *et al.*, 2015). This technique involves a DNA polymerase and a set of four specially designed primers that identify six distinct sequences on the target

Table 4. A summary of representative studies evaluating the performance of real-time PCR and other methods.

| No | Country | Study population | Sensitivity | | References |
|---|---|---|---|---|---|
| | | | qPCR | Other methods | |
| 1 | Ethiopia | 844 school children | Sensitivity: *S. stercoralis* 73.9% | **Formol-Ether concentration Technique (FECT):** *S. stercoralis* 5.2%<br>**Spontaneous tube sedimentation technique (STST):** *S. stercoralis* 10.3%<br>**Baermann concentration technique (BCT):** *S.stercoralis* 26.4%<br>**Agar plate culture (APC) :** *S. stercoralis* 28.0% | (Hailu *et al.*, 2022) |
| 2 | Fiji | 40 individuals | Sensitivity qPCR compare to Kato-Katz: *A. lumbricoides* 75% and hookworms 66.7% | | (Azzopardi *et al.*, 2021) |
| 3 | Tanzania | 1636 individuals | Sensitivity: *T. trichiura* 94.4%, *A. lumbricoides* 85.1%, and hookworm 85.2% | **Kato-Katz:** *T. trichiura* 78.2%, *A. lumbricoides 38.5%* and hookworm 39.7% | (Keller *et al.*, 2020) |
| 4 | Bangladesh | 2,799 children | Sensitivity*: A. lumbricoides* 79%, hookworm 93%, and *T. trichiura* 90% | **Double-slide Kato-Katz:** *A. lumbricoides* 49%, hookworm 32% and *T. trichiura* 52% | (Benjamin-chung *et al.*, 2020) |
| 5 | Tanzania | 320 individuals | Sensitivity: *T. trichiura* 89.1%, hookworm 72.7% and *A. lumbricoides* 87.5% | **Kato-Katz:** *T. trichiura* 83.6%, hookworm 43.0% and *A. lumbricoides* 53.8% | (Barda *et al.*, 2020) |
| 6 | Brazil, Ethiopia, Lao PDR, and Tanzania | 645 children | Sensitivity: *A. lumbricoides* 90.0%, *T. trichiura* 94.7% and hookworm 91.9% | **Single Kato-Katz:** *A. lumbricoides* 71.9%, *T. trichiura* 88.1% and hookworm 72.6%<br>**Duplicate Kato-Katz:** *A. lumbricoides* 73.0%, *T. trichiura* 90.9% and hookworm 75.3%<br>**Mini-FLOTAC:** *A. lumbricoides* 63.3%, *T. trichiura* 91.5% and hookworm 73.9%<br>**FECPAK$^{G2}$** *A. lumbricoides* 58.9%, *T. trichiura* 59.8% and hookworm 52.4% | (Cools *et al.*, 2019) |
| 7 | Timor Leste | 571 children (860 sample) | Sensitivity: *A. lumbricoides* 94.1%, hookworm 75.7%, and *T. trichiura* 53.1% | **Sodium nitrate flotation (SNF):** *A. lumbricoides* 68.1%, hookworm 66.9% and *T. trichiura* 81.3% | (Clarke *et al.*, 2018) |

| | | | | | |
|---|---|---|---|---|---|
| 8 | Philippines | 263 school children | Sensitivity: *A. lumbricoides* 60.8%, and *T. trichiura* 38.8% | **Kato-Katz:** *A. lumbricoides* 20.5%, and *T. trichiura* 23.6% | (Mationg *et al.*, 2017) |
| 9 | Kenya | 796 individuals | Sensitivity: *A. lumbricoides* 98% and *N. americanus* 98% | **Kato-Katz:** *A. lumbricoides* 70% and *N. americanus 32%* | (Easton *et al.*, 2016) |
| 10 | Côte d'Ivoire | 256 individuals | Sensitivity: *S. stercoralis* 76.8% | **Koga agar plate:** *S. stercoralis* 21.4%<br><br>**Baermann:** *S. stercoralis* 37.5% | (Becker *et al.*, 2015) |

DNA (Notomi *et al.*, 2000). LAMP has been suggested by Ngari and co-workers (2020) as potential rapid diagnostics for *T. trichiura*, both in laboratories and field setups, because of its convenience, low cost, high sensitivity, and specificity. Rashwan and co-worker (2017) recently developed a colourimetric isothermal assay using asymmetrical SmartAmp2 primer sets and reagents in LAMP to identify *N. americanus*, *T. trichiura*, and *A. lumbricoides* in human faecal samples. The assay involves a species-specific primer-specific target sequence on the *β*-tubulin gene for the detection of different species (Manuel *et al.*, 2021)

**Recent Technical Developments**

A quantitative paper-based DNA reader (qPDR) is recently developed to quantify STH at the molecular level by measuring distance as readout. Thus, external readers are not required. Based on the unique interfacial interaction of a DNA intercalating dye SYBR Green I with native cellulose on a chromatographic paper, the distance-based quantification of minute amounts of double-stranded DNA as short as 6 min can be performed with qPDR. qPDR can be integrated with PCR with the aid of a smartphone-controlled portable thermal cycler to quantify a minute amount of genetic markers from adult worms of an STH. It has been successfully used to demonstrate that *T. trichiura* was expelled post-treatment in infected children living in the rural areas of Honduras (Wang *et al.*, 2018).

Cell-free DNA assays are developed to detect DNA released into the bloodstream or appearing in the urine as cells pass through the glomerular barrier or found in other body fluids like sputum, saliva, or stool (Manuel *et al.*, 2021). This method can also be used to detect STH through the cell-free DNA from the disintegration or decay of the parasites during different stages of their life cycle (Weerakoon & McManus, 2016). A study in Iran demonstrated successful detection of Strongyloides infection in immunosuppressed patients using cell-free DNA from serum (Gorgani-Firouzjaee *et al.*, 2018). Another study conducted in Argentina showed successful detection of *S. stercoralis* using urine samples, and it was found to have a significant advantage over faecal examination (Lodh *et al.*, 2016). Thus, this technique is worth to be explored further for STH detection (Manuel *et al.*, 2021).

**Limitations of molecular-based techniques**

There are many limitations associated with molecular-based techniques. Stool samples contain highly variable components dependent on nutrition, gut flora, lifestyle, and environment of the patient (Schrader *et al.*, 2012) that may inhibit molecular reaction (Khurana & Sethi, 2017). Inhibitors may include polysaccharides or chlorophyll originating from herbs and vegetables, bile salts, urea, glycolipids, haemoglobin and heparin (Schrader *et al.*, 2012). The major consequence of partial or total inhibition of PCR reactions is decreased sensitivity or false-negative results, respectively (Schrader *et al.*, 2012). Therefore, it is crucial to eradicating inhibitors through an established extraction protocol, achievable with many commercial nucleic acid extraction kits developed with inhibitor removal steps (Papaiakovou *et al.*, 2019; Khurana & Sethi, 2017).

Besides, the shells of STH eggs are much more rigid than the cell walls of bacteria, thus causing a lower yield of nucleic acid (Khurana & Sethi, 2017). Several mechanical procedures (extensive heating, vortexing, blending and sonication), chemical procedures (alkaline supplementation, addition of lyticase, achromopeptidase or a higher amount of proteinase K) and a few combinations of mechanical and chemical procedures were evaluated for enhanced release of egg DNA. Still, none of them was promising (Kaisar *et al.*, 2017). The bead-beating method before DNA extraction has been confirmed to destroy the eggshells and led to increased DNA yield in human faecal samples (Ayana *et al.*, 2019; Kaisar *et al.*, 2017). Although various types of beads, such as glass, garnet, zirconium, ceramic, and steel, have been investigated, their impact on detection sensitivity and DNA concentration has yet to be determined (Ayana *et al.*, 2019).

Another challenge is obtaining worm eggs to be used as controls (Azzopardi *et al.*, 2021). Many eggs at varying development stages for each STH species and labour-intensive processes for the purification, picking and placing of eggs are required for assured

quality of reference (Cools *et al.*, 2021; Baylis *et al.*, 2019). An accessible distributed network connecting active researchers to update relevant research and reference material for further STH diagnostic development is necessary for information sharing (Papaiakovou *et al.*, 2021; Emery *et al.*, 2012). Besides a biobank or a global bank of eggs, larvae, and DNA must be made available to serve as a reference for wide implement qPCR to diagnose these STH (Azzopardi *et al.*, 2021).

The lack of standardisation challenges the general adoption of qPCR diagnostics (Stuyver & Levecke, 2021). The existing methods to preserve stool samples, DNA extraction and qPCR protocols show varying efficiencies (Cools *et al*., 2021). Since immediate DNA isolation from fresh stool samples is impossible in the field, rapid freezing of the samples should be conducted to prevent DNA degradation from the nucleases found within faeces (Papaiakovou *et al*., 2018). Besides, internal amplification controls to determine the robustness of the DNA extraction process and standards of different STH species are required to establish qPCR protocols (Easton *et al*., 2016). Complete annotation of STH genomes is an ongoing process to use plasmids as standards where plasmids are potentially more cost-effective and feasible than worm material (Cools *et al*., 2021). Standardised validation and assessment of infection intensity using qPCR are also required as DNA concentrations are commonly calculated based on individual standard curves. Different standard curves are used as quantitative measures of infection intensity (Le B *et al*., 2022; Papaiakovou *et al*., 2018).

**Operational Cost and Infrastructure**

The accuracy of diagnostic tools plays a pivotal role in an epidemiological study, monitoring treatment efficacies in mass drug administration (MDA) programs and the possibility of drug-resistance development (Sanprasert *et al.,* 2019). However, the choices of a diagnostic technique are commonly based on cost and simplicity, with little emphasis on their sensitivity (Bergquist *et al*., 2009). Microscopy methods are preferred over molecular-based techniques due to their low cost. Although molecular diagnostics such as qPCR provides sufficient sensitivity, specificity, and throughput, their higher costs hinder their comprehensive implementation in most endemic regions where widespread poverty and resources are limited (Wang *et al*., 2018).

In addition, sophisticated equipment required for molecular-based diagnosis is not particularly suitable for field surveys or rapid identification for urgent treatment due to the frangibleness of the machines and the longer time needed for diagnosis (Ngwese *et al.,* 2020). Moreover, tropical communities of low socioeconomic status lack the required technology, resources, and expertise to access the requisite facilities (Weerakoon & McManus, 2016). Although the significant drawback of molecular-based techniques such as qPCR is their high cost, lowering their price to a competitive level is theoretically possible. However, an upgrade in technology, infrastructure, financial support and expertise is required to implement molecular-based techniques in endemic countries (O'Connell & Nutman, 2016; Stuyver & Levecke, 2021).

**Conclusion**

In conclusion, molecular-based methods are more sensitive, rapid, and specific for STH detection than the microscopy based-method. Among various molecular diagnostics, qPCR excels with its capability to diagnose mixed infections simultaneously in a short time. Besides, the high reproducibility of qPCR results obviates the need for multiple sampling and reagents reduction to save cost. However, there are currently no standard guidelines and procedures for DNA extraction and sample processing for STH samples before qPCR diagnosis. Therefore, attention from WHO and researchers is required to develop new standard guidelines to ensure a reliable and efficient alternative diagnostic to the conventional method. Thus, standardisation followed by the implementation of qPCR in STH epidemiology and control programs for case reporting is highly advised. In addition, a standard procedure is also necessary to achieve uniformity in faecal samples preservation, DNA extraction, and qPCR settings to assure results reproducibility in the studies. Moreover, the quality of diagnosis is limited as stool samples cannot be stored. Thus, the samples must be processed in a minimum time to assure the accuracy of diagnosis.

Microscopy is still reliable with advancements offered, such as the FECPAK$^{G2}$. However, this assay is less sensitive than single and two-slide Kato-Katz for human samples. Therefore, it is required to increase the sensitivity and egg recovery for FECPAKG2 to be a reliable diagnostic tool for microscopic detection, particularly for field applications.

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**Conflict of Interest**

Authors declare no conflict of interest.

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