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Review

Molecular Tools for Diagnosis and Surveillance of Soil-Transmitted Helminths in Endemic Areas

Malathi Manuel, Karthik Ramanujam and Sitara S. R. Ajjampur *

The Wellcome Trust Research Laboratory, Division of Gastrointestinal Sciences, Christian Medical College, Vellore 632004, Tamil Nadu, India; malathimanuel25@gmail.com (M.M.); karthikjpt@gmail.com (K.R.)
* Correspondence: sitararao@cmcvellore.ac.in; Tel.: +91-416-228-2052

Abstract: Soil-transmitted helminths (STH) including the hookworms *Necator americanus* and *Ancylostoma* spp., *Ascaris lumbricoides*, and *Trichuris trichiura* affect over 1.5 billion people worldwide and are estimated to have caused 1.9 million disability-adjusted life years (DALYs). With the concerted effort in expanding and improving targeted mass drug administration (MDA) programs over the past decade, along with decreasing prevalence, infections in several endemic areas tend to be of low intensity. Conventional microscopy-based methods recommended for the detection of STH in parasitological surveys have been shown to be less sensitive in these low-intensity settings. As communities progress towards STH elimination through MDA and improved sanitation, there is a pressing need for highly sensitive techniques that detect the true prevalence of STH to evaluate the effectiveness of ongoing programs and interventions. Molecular methods that involve analysis of DNA rather than the morphology of the organism are highly sensitive and specific, allowing for both quantitation and species discrimination. The following review discusses different sample collection strategies, pre-processing steps, DNA extraction platforms, and nucleic acid detection methods available for diagnosis and surveillance of STH. We have contrasted the utility of these molecular tools against conventional microscopy-based methods currently used in most endemic settings. While the detection methods are primarily qPCR based, several newer technologies have also become available along with automation and increased throughput, making these molecular tools increasingly cost-effective and potentially amenable for use in low-resource settings.

Keywords: soil-transmitted helminths; hookworm; *Ascaris lumbricoides*; *Trichuris trichiura*; PCR

1. Introduction

Soil-transmitted helminths (STH) affect an estimated one billion of the world's population (772–892 million with *Ascaris lumbricoides*, 430–508 million with *Trichuris trichiura*, and 406–480 million with hookworm) [1]. STH infections can lead to adverse health issues such as chronic blood loss leading to anemia, malabsorption of nutrients, loss of appetite, stunting, lethargy, school absenteeism, and lower performance on a range of cognitive tests [1–4]. In order to reduce STH-related morbidity, the WHO recommends annual or biannual targeted deworming of high-risk populations, including pre-school aged children (PSAC), school-aged children (SAC), and women of reproductive age (WRA) [5]. The frequency of deworming required in a region or country is based on the prevalence and intensity of infection, which is estimated by population-level parasitological surveys (usually carried out in school children) [6]. In these surveys, a known volume of a stool sample is tested for the presence of STH ova, and the worm burden (eggs per gram, EPG) for each species is estimated using microscopy-based methods. The EPG data are then used to classify infections into categories: heavy, moderate, and light intensity, for each species [7].

Several microscopy-based techniques for the detection of STH in both diagnostic laboratories and epidemiological surveys have been developed (Table 1). While direct wet mount preparation and fecal concentration methods (like formol–ether sedimentation and zinc sulfate floatation) remain the primary diagnostic tests for parasites in many laboratories, studies have shown these techniques to be less sensitive than the Kato–Katz method [8,9]. The Kato–Katz method, which also provides the EPG, is currently the most commonly used technique in population-based prevalence surveys but shows better sensitivity for *A. lumbricoides* and *T. trichiura* detection than hookworm [10]. The poorer sensitivity of this method for the detection of hookworm is due to the rapid disintegration of the ova [11]. The McMaster technique, originally used in veterinary samples, was shown to have an equivalent sensitivity to Kato–Katz for the detection of hookworm and *T. trichiura* with a good correlation of egg counts between these two techniques in a multicountry comparison [12]. This technique is relatively easy and less time-consuming. The major drawback with both the Kato–Katz and McMaster methods compared to other microscopy-based methods is their high limits of detection (LODs) of 24 EPG and 50 EPG, respectively. The FLOTAC method, which can be used to enumerate egg counts from a larger volume of fecal material (1 g or more), has a lower LOD of 1–2 EPG. This technique, however, requires a centrifuge, which can be a constraint in resource-limited settings and field surveys [13]. Although this technique has a lower LOD than Kato–Katz, it was shown to underestimate EPG counts [14,15]. The mini-FLOTAC is a simplified form of FLOTAC that does not require costly equipment. A meta-analysis indicated a sensitivity comparable to Kato–Katz for the detection of STH [16]. The FECPAK^{G2}, a recently developed image-based egg counting method, was also less sensitive than single and two-slide Kato–Katz [17]. Lab-on-a-disk is another newly developed egg quantification platform that involves floatation followed by centrifugation and imaging, and showed a good correlation with the mini-FLOTAC when evaluated using samples from pigs and humans in Ethiopia [18]. Point-of-care mobile diagnostics with digital microscopy and deep learning-based image analysis algorithms have also been evaluated in proof-of-concept studies but need to be evaluated more widely in field settings [19,20].

Table 1. Microscopy-based detection methods for soil-transmitted helminths.

Method	Principle	Sensitivity	Negative Predictive Value	Reference
Wet mount preparation	Preparation of stool sample with saline/iodine on a microscopic slide with a cover glass.	Hookworm: 85.7% *A. lumbricoides*: 83.3%	Hookworm: 97.5% *A. lumbricoides*: 98.8%	[8]
		Hookworm: 37.9% *A. lumbricoides*: 52% *T. trichiura*:1 2.5%		[9]
Formol–ether sedimentation	Stool sample diluted in distilled water is centrifuged with 3% ethyl ether. Of the four layers formed, the lower sediment is mixed with 5% formalin and 50 uL of this sediment screened under a microscope.	Hookworm: 95.8% *A. lumbricoides*: 94.2% *T. trichiura*: 86.7%	Hookworm: 98.8% *A. lumbricoides*: 98.5% *T. trichiura*: 99.8%	[21]
		Hookworm: 72.4% *A. lumbricoides*: 81.4% *T. trichiura*: 57.8%		[9]

Table 1. *Cont.*

Method	Principle	Sensitivity	Negative Predictive Value	Reference
Kato–Katz	A known amount of sieved stool sample is placed on a glass slide and covered with cellophane soaked in methylene blue solution and screened under a microscope after 30 min and the eggs are counted.	Hookworm: 69% *A. lumbricoides*: 93.1% *T. trichiura*: 90.6%		[9]
		Hookworm: 72.4% *A. lumbricoides*: 57.4% *Trichuris* spp.: 84.4%		[10]
		Hookworm: 62.9% *A. lumbricoides*: 72.4% *T. trichiura*: 95%	Hookworm: 58.8% *A. lumbricoides*: 81.3% *T. trichiura*: 50%	[22]
		Hookworm: 81% *A. lumbricoides*: 68% *T. trichiura*: 88%		[15]
		A. lumbricoides: 84.4%	*A. lumbricoides*: 96.7%	[23]
		Hookworm: 89.1% *A. lumbricoides*: 89.8% *T. trichiura*: 96.1%		[17]
		Hookworm: 19.6% *A. lumbricoides*: 67.8% *T. trichiura*: 76.6%	Hookworm: 84.3% *A. lumbricoides*: 86.7% *T. trichiura*: 75.3%	[14]
		Hookworm: 93.9% *A. lumbricoides*: 96.2% *T. trichiura*: 98.3%		[24]
		Hookworm: 95.1% *A. lumbricoides*: 97.3% *T. trichiura*: 86.7%	Hookworm: 98.6% *A. lumbricoides*: 99.3% *T. trichiura*: 99.8%	[21]
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 eggs are counted.	Hookworm: 67.6% *A. lumbricoides*: 74.3% *T. trichiura*: 94.6%	Hookworm: 62% *A. lumbricoides*: 82.4% *T. trichiura*: 47.8%	[22]
		Hookworm: 92.3% *A. lumbricoides*: 94.9% *T. trichiura*: 95.2%		[24]
		A. lumbricoides: 48.3%	*A. lumbricoides*: 91.5%	[23]
		Hookworm: 80.8% *A. lumbricoides*: 69.5% *T. trichiura*: 46.7%	Hookworm: 94.6% *A. lumbricoides*: 92.7% *T. trichiura*: 99.4%	[21]
FLOTAC	A weighed 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 two FLOTAC chambers. The chambers are centrifuged and examined under a microscope.	Hookworm: 80.8% *A. lumbricoides*: 81.9% *T. trichiura*: 96.8%	Hookworm: 73.4% *A. lumbricoides*: 86.9% *T. trichiura*: 61.1%	[22]
		Hookworm: 54% *A. lumbricoides*: 88% *T. trichiura*: 95%		[15]
		Hookworm: 100% *A. lumbricoides*: 100% *T. trichiura*: 100%	Hookworm: 100% *A. lumbricoides*: 100% *T.t richiura*: 100%	[14]
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 chamber.	*A. lumbricoides*: 61.3%	*A. lumbricoides*: 93.1%	[23]
		Hookworm: 70.8% *A. lumbricoides*: 42.1% *T. trichiura*: 85.6%		[10]
FECPAK[G2]	FECPAK[G2] platform with a cassette, concentrates helminth eggs into one microscopic field of view and this is photographed and is stored on a computer from which the eggs can be counted.	Hookworm: 47.5% *A. lumbricoides*: 36.8% *T. trichiura*: 37.5%		[10]
		Hookworm: 91.3% *A. lumbricoides*: 96.9% *T. trichiura*: 95.3%		[17]

Studies have shown that the rate of egg excretion varies from day to day and hence even the best techniques that rely on microscopy can result in false-negative results [25,26].

Population-based surveys from areas with a predominantly light intensity of infection have shown that standard microscopy-based techniques are suboptimal [27–29]. Additionally, the cost of Kato–Katz, while being perceived as cheap, may be higher and more variable when more samples or survey sites are added [30]. Hence, there is a need to develop more sensitive, less labor-intensive, and high-throughput detection methods. Furthermore, these methods should be cost-effective and able to be used in large-scale national surveys to monitor ongoing programs and measure the impact of interventions in endemic settings [31–33]. The cost of these newly developed techniques may remain higher than Kato–Katz or other microscopy-based methods, however, the benefits of increased accuracy and sensitivity may outweigh the additional cost [30].

In this review, we discuss the advantages and limitations of the various molecular tools used for STH detection in comparison to the conventional microscopy-based methods used in endemic settings. We have also described in detail the sample collection, storage, and pre-processing steps required for accurate nucleic acid detection and quantitation.

2. Role of Molecular Methods

Molecular methods have significantly improved the sensitivity of detection and quantitation of STH. They are also highly specific as molecular methods can help distinguish between closely related, morphologically similar, human and non-human (zoonotic) species compared to microscopic methods which could misidentify pathogens [27,29]. Some of the different molecular assays used for STH diagnosis are PCR, including real-time PCR and digital PCR, isothermal assays (LAMP), and, more recently, cell-free DNA detection (Table 2). The DNA sequences that have been targeted for the detection of STH include ribosomal DNA [34], repetitive sequences [35], and mitochondrial genes [36]. The rDNA genes and internal transcribed spacers (ITSs) are clusters of tandem repeats. They are targets of choice to detect morphologically similar parasites as they have sufficient heterogeneity and are found to exist as moderate copy number repeats [37]. The mitochondrial genes, especially cytochrome oxidase-I, have also been widely used for the detection of parasites and are more conserved targets than nuclear DNA [38]. More recently, Pilotte et al. developed a PCR that targets non-coding, repetitive DNA sequences using a next-generation sequencing approach that identifies genome-wide eukaryotic repetitive elements [35]. Targeting repetitive DNA sequences that make up several thousand to a million copies per diploid genome can greatly improve sensitivity with the lowest limit of detection being at or above 2 fg/uL, which is less than the quantity of DNA present in a single STH egg [35,39].

Table 2. Molecular detection methods for soil-transmitted helminths.

Molecular Technique		Target	STH Detected	Sensitivity *	Negative Predictive Value *	Reference
Conventional PCR	Single-plex	Mitochondrial COI gene	*A. duodenale, N. americanus*			[36]
Nested PCR	Semi-nested PCR	ITS-2	*A. duodenale*			[40]
		28S rRNA, ITS-2	*N. americanus*	*N. americanus*: 94.5%		[41]
	Semi-nested PCR+ RFLP	ITS-1, 2 and 5.8S region	*A. duodenale, N. americanus,*			[42]
			T. trichiura, A. lumbricoides			
Quantitative paper-based DNA reader	Single-plex mini-PCR	β-tubulin	*T. trichiura*			[43]

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Table 2. *Cont.*

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Molecular Technique		Target	STH Detected	Sensitivity *	Negative Predictive Value *	Reference
Real-time PCR	Single-plex	ITS-2	*A. duodenale, N. americanus*	Hookworm: 78.9%		[44]
		Repetitive sequence	*N. americanus, A. lumbricoides, T. trichiura, A. duodenale*			[35]
	Multiparallel	ITS-1,2	*A. lumbricoides, T. trichiura, A. duodenale, N. americanus*		*A. lumbricoides*: 96.7% *T. trichiura*: 99.2%	[45]
		Repetitive sequences, ITS-1,2	*A. lumbricoides, T. trichiura, A. duodenale, N. americanus*	*A. lumbricoides*: 98% *N. americanus*: 98%		[28]
	Multiplex	ITS-2	*A. duodenale, N. americanus*			[34]
		ITS-1,2	*A. lumbricoides, Ancylostoma* spp., *N. americanus*			[46]
		ITS-1,2	*N. americanus, T. trichiura, Ancylostoma* spp., *Ascaris* spp.			[47]
	PCR-Luminex	ITS-1,2	*N. americanus, A. lumbricoides, A. duodenale*			[48]
	Melt curve analysis	ITS-2	*N. americanus, A. duodenale, A. ceylanicum, A. caninum, A. braziliense*	Hookworm: 100%		[49]
		18S, ITS-1,2	*A. lumbricoides, T. trichiura, A. duodenale, N. americanus*			[50]
	Multiplex-tandem PCR–qPCR	β-tubulin	*A. lumbricoides, T. trichiura, A. duodenale, N. americanus*			[51]
Digital PCR		ITS-1	*A. lumbricoides*			[52]
Isothermal assay	SmartAmp2	β-tubulin	*N. americanus, A. lumbricoides, T. trichiura*			[53]
	LAMP	ITS-2	*N. americanus*	*N. americanus*: 97%		[54]
	LAMP	ITS-1	*A. lumbricoides*	*A. lumbricoides*: 96.3%	*A. lumbricoides*: 88.9%	[55]

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* Compared to microscopy.

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3. Sample Storage and DNA Extraction Methods

Sample storage and DNA extraction are critical steps in any molecular assay. Papaiakovou et al. compared standard sample storage at −20 °C used in most laboratories with different sample preservation techniques, including a silica bead two-step desiccation process, FTA cards, 5% potassium dichromate, RNAlater, ethanol, Paxgene, and Formalternate, and stored them at two different temperatures (4 °C and 32 °C) for 60 days. This study was carried out using stool samples spiked with *N. americanus* ova. They found that while all preservation techniques were stable at 4 °C, only storage using the silica bead two-step

desiccation process, 5% potassium dichromate, and FTA card-based preservation had good stability at 32 °C with no significant change in the concentration of DNA detected [56]. In another study, storage with 96% ethanol at 4 °C was found to detect more concentrations of DNA than potassium dichromate and RNAlater [57]. Replicating these types of studies in real-world endemic settings will help determine alternative approaches for storage and transport of samples. This would be especially useful for surveys in remote or resource-limited settings where setting up a cold chain may not be feasible due to high costs and lack of infrastructure.

Over the years, there have been several advancements in DNA extraction methods for STH ova. Manual extraction followed by commercially available kit-based extractions that vary based on the sample type, to automated extraction platforms have been described. In the case of STH, it is essential to employ steps that homogenize fecal samples and break up the outer layer of the ova to obtain the maximum DNA yield. The commonly used egg disruption steps for fecal homogenates involve heating at high temperatures, multiple freeze–thaw cycles, and bead beating using non-degradable beads like silica, zirconium, etc. Several different lysis protocols have been developed, including: (1) washing with PBS then re-suspending in 2% polyvinylpolypyrrolidone (PVPP) and heating at 100 °C for 10 min [34], (2) mixing with lysis buffer, proteinase K, and β-mercaptoethanol, then freezing at 80 °C for 30 min followed by heating at 60 °C for 2 h [58], (3) multiple freeze–thaw cycles from liquid nitrogen to a water bath at 95 °C [42], (4) bead beating with ceramic beads [45], and (5) adding 2% PVPP followed by freezing overnight at −20 °C and then bead beating [59]. Ayana et al. compared two commercially available kits with and without a prior bead-beading step and found that bead beating significantly increased DNA yield [57]. Most laboratories now prefer commercially available kit-based DNA extraction methods but these also require pre-processing steps such as bead beating and/or lysis. The different DNA extraction kits evaluated for STH include Fecal Isolate II DNA Extraction Kit (Bioline, London, UK) [60], Powersoil DNA Isolation Kit (MO Bio, Carlsbad, CA, USA) [47], FastDNA Spin Kit for Soil (MP Biomedicals, Irvine, CA, USA) [61], QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA, USA) [59], DNeasy Tissue Kit (Qiagen, Hilden, Germany) [62], and the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) [63]. Automated platforms have also been used for STH, including Automate Express[TM] (Applied Biosystems[TM], Waltham, MA, USA) [64] and the Easy-plex system (AusDiagnostics, Mascot, Australia) [65], but remain challenging to establish in low-resource settings due to the infrastructure required.

4. Conventional PCR

The polymerase chain reaction, PCR, has significantly improved the detection of STH, showing greater sensitivity and detecting DNA at very low concentrations. A study that compared PCR with the formalin–ethyl acetate concentration technique for the detection of *A. lumbricoides*, *N. americanus*, and *S. stercoralis* in stool samples found that PCR was able to detect DNA concentrations as low as 0.001 ng [66]. Conventional PCR has also been used with other downstream techniques such as restriction fragment length polymorphism (RFLP) or Sanger sequencing to identify different species of STH [42]. Alternatively, nested or semi-nested PCRs to detect different species of the same genus have been carried out, for example, a study in a rural community in Thailand used a semi-nested approach to detect two different species of *Trichuris*—namely *T. trichiura* and *T. vulpis* [67]. PCR has also been multiplexed to detect multiple organisms within the same reaction, thereby reducing cost. This was evident in another study in Thailand where they used a triplex PCR approach to detect *A. lumbricoides, N. americanus*, and *T. trichiura* in stool samples. Their findings showed a combined STH sensitivity and specificity of 87% and 83%, respectively, with the ability to detect a single copy of DNA [68]. The major drawback with conventional PCR is the need for post-PCR analysis, which increases cost, turn-around time, and has a potential for contamination. With the availability of qPCR, conventional PCR is not commonly used for the detection of STH.

5. Real-Time PCR

Real-time PCR or quantitative PCR (qPCR) has been widely used for the detection of STH by several research groups (Table 2). qPCR assays can detect DNA concentrations that are lower than the amount of DNA found in a single fertilized STH ovum [69] and outperform Kato–Katz sensitivity and specificity even in endemic settings [28]. Two different chemistries, namely, DNA intercalating dyes like SYBR green [70] or probe-based assays like TaqMan, have been developed for STH detection, with the latter allowing for multiplexing.

Some of the real-time PCR-based techniques that have been used for the detection of STH include PCR-Luminex, high-resolution melt curve analysis, and multiplexed-tandem PCR. A PCR-Luminex assay (binding of PCR products to carboxylated Luminex beads with biotinylated primers and amine-modified probes) is a technique that has shown to be a high-throughput alternative to microscopy, with sensitivity and specificity comparable to qPCR [48]. Real-time PCR coupled with high-resolution melt curve analysis is another technique that has been used for the detection of different species of hookworm [49]. Multiplexed-tandem PCR is a newly developed technique that involves a multiplex, conventional PCR followed by a tandem real-time PCR. After this, the amplicons are subjected to a high-resolution melt curve analysis [51]. Detection of multiple helminths species by real-time PCR is, however, more often carried out with either a multiplex or a multiparallel approach.

Multiplex PCR has been shown to be more sensitive than microscopic techniques, including Kato–Katz, for nearly all STH with an LOD of 10 copies [46]. The multiparallel qPCR approach, where different species are tested in different reaction tubes, has a higher sensitivity than multiplex PCR, allowing detection of low-intensity infections (which may remain undetected in multiplex reactions). Another advantage of the multiparallel format is that it provides greater flexibility in selecting species-specific PCRs tailored to the endemicity in a particular region [45]. qPCR has also showed a better sensitivity when used to assess the efficacy of anthelminthic drugs, as it was able to detect infections that were undetected by Kato–Katz due to lower worm burden [71].

qPCR, as the name suggests, has also been employed for the quantification of STH infection. Easton et al. compared the EPG determined by Kato–Katz in rural Kenya with DNA concentration derived from qPCR for *A. lumbricoides* (ITS primers) and *N. americanus* (repetitive sequence primers) and found a strong correlation between EPG and average DNA concentration [28]. Mejia et al. also compared Kato–Katz and qPCR (ITS primers) and found a good correlation between egg count and DNA concentration for *A. lumbricoides* and *T. trichiura* [45]. A similar strong correlation was found between Kato–Katz EPG and DNA copy number (repetitive sequence primers) for *A. lumbricoides* and *T. trichiura* [72], whereas a study by Barda et al. in Tanzania showed poor correlation between EPG and DNA copy number for hookworm and moderate correlation for *T. trichiura* and *A. lumbricoides* [73]. Other groups have correlated EPG with raw qPCR Ct values rather than DNA concentration or copy number. A multiplex qPCR targeting *N. americanus, A. duodenale*, and *O. bifurcum* carried out in three sites in Africa showed that Ct values for *N. americanus* (ITS primers) had a good linear correlation with Kato–Katz EPG [34] while a study from Bangladesh showed a moderate correlation between EPG (double-slide Kato–Katz) and Ct values (repetitive sequence primers) [29]. A study by Llewellyn et al. used a multiplex qPCR assay (ITS primers) for the detection of *Ascaris* spp., *Ancylostoma* spp., *N. americanus*, and *T. trichiura* and converted Ct values to EPG and found a strong linear relationship [47].

Most studies have shown a correlation between DNA concentration or Ct values with EPG. However, when qPCR was used to categorize the intensity of infection into light, moderate, or heavy infections to provide data that are both programmatically and clinically relevant, mixed results for each species were obtained. A recent study found that although the median Ct values of samples which were microscopy negative or of light intensity (as classified by Kato–Katz for *A. lumbricoides*) were higher than for samples classified as MHI, there was overlap in the range of Ct detected [29]. A study by Knopp et al. found no

difference in Ct values for hookworm when Kato–Katz was positive or negative [27]. A recent study was conducted by Levecke et al. using samples from multiple sites to compare categorization of infection intensity between multiple microscopy-based methods and qPCR. They found that that although ~80% of samples were correctly categorized as MHI by qPCR, an additional 10–25% of light-intensity infections (depending on species) were categorized as MHI by qPCR.

Data across these studies are, however, difficult to compare due to the different units of measurement used to represent intensity along with the different methods used [74]. In 2018, an international external quality assurance scheme for STH was initiated as a step towards more uniform reporting. The scheme was for nucleic acid amplification tests and has helped validate the diagnostic performance of these laboratories as well as allowed for comparability [75]. Another suggestion for more uniform reporting has been to express qPCR results more uniformly, in genomic equivalents per mL (GE/mL), to overcome issues such as variable primers and target copy numbers [76].

While the Ct value can be used to determine the infection intensity, multiple factors need to be taken into account while using qPCR as a quantitation method for STH ova. As Papaiakovou et al. mention, it is important to consider that in STH ova, haploid, diploid, or developing larval stages can be present. The copy number of the target sequences also needs to be considered, especially while using multicopy regions, which can improve sensitivity of detection but can lead to over- or under-quantification [77].

Various studies have developed qPCR with very low reaction volumes and improved reagents, significantly reducing the cost of detection. Mejia et al. estimated that a small reaction volume qPCR for the detection of eight parasites in a stool sample costs less than USD 1 [45]. Pooled testing of samples has also been evaluated as a cost-effective method [78,79] but the ability to detect a positive infection decreases when the intensity is low [80].

6. Isothermal Assay (LAMP)

The loop-mediated isothermal assay developed by Notomi et al. [81] works on the principle of auto-strand displacement DNA synthesis. Due to the isothermal conditions, the assay only requires a water bath or thermal block, thereby reducing the cost, and the reaction can be colorimetrically visualized. Two studies that compared LAMP with Kato–Katz for the detection of *N. americanus* and *A. lumbricoides* found the sensitivity of LAMP to be over 95% but with variable specificity [54,55]. The Smart Amplification2 (SmartAmp2) assay works in isothermal conditions and uses asymmetrical primers that allow more specific detection of various organisms. This has been applied to STH by Rashwan et al, who found that it allows detection of as little as 1 pg genomic DNA within 50–60 min [53]. The same group also developed a SmartAmp2 assay for the detection of benzimidazole resistance in *N. americanus* that target codons 167, 198, and 200 in the β-tubulin isotype 1 gene [82].

7. Other Recent Technical Developments

Digital PCR is a recently developed technique used for the detection of various parasitic infections [83]. It allows partitioning of a DNA sample into several individual reactions, with some reactions containing the target molecule, and others being negative. A single molecule is amplified a million-fold or more with TaqMan chemistry. The fraction of negative reactions is used for the absolute quantification of the total target molecule in a sample. It allows for quantitation without the need for a standard curve or endogenous controls. When the LODs of qPCR and digital PCR for the detection of *A. lumbricoides* egg in reclaimed water were compared, both techniques were able to detect one *A. lumbricoides* egg in 500 mL of reclaimed water [52]. Droplet digital PCR (ddPCR) is a form of digital PCR that is based on oil–water emulsion technology for the partitioning of the reaction mixture and is currently being evaluated for detection of STH in environmental samples in endemic areas as a potential surveillance tool.

In cell-free DNA assays, 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 is detected. Detection of the pathogen in urine and sputum can be less invasive, simple, and cost-effective when conducted at a large scale and has been used for the detection of *Leishmania*, *Plasmodium*, *Trypanosoma*, *Schistosoma*, and microfilaria. This method can also be used for the detection of STH due to the presence of cell-free DNA from the disintegration or decay of the parasites during different stages of their life cycle [84]. A study conducted in Iran used cell-free DNA from serum to detect *Strongyloides* infection in immunosuppressed patients [85]. Another study in Argentina detected *Strongyloides* in urine samples and it was found to have a significant advantage over fecal examination [86]. Thus, this technique can potentially be explored for STH detection.

A new field-adapted method has been developed recently using miniPCR®. Here, genomic DNA is amplified with a portable thermocycler, after which the amplicon is loaded on a quantitative paper-based DNA reader which quantifies DNA based on the distance traveled on a cellulose strip which is then visualized for the naked eye [43]. This fast and relatively simple method can be used in field settings as a point-of-care (POC) test but requires validation and development of an equally portable stool DNA extraction method.

8. Conclusions

Molecular techniques are highly sensitive and specific for the detection of STH and can also detect benzimidazole resistance markers. A current limitation of molecular tools is the difficulty in determining the intensity of infection to provide relevant programmatic data. Limitations also include initial cost, and requirement for specialized equipment (in most methods), infrastructure, and trained personnel, thus making them difficult to be implemented in resource-limited settings. However, when combined with high-throughput automation at scale, molecular methods could potentially replace the more human resource-intensive microscopy-based assays in national-level survey programs. In this scenario, small-volume PCR reactions and pooled testing would also contribute to reduced costs. Issues like stability of samples and requirements for an expensive cold chain can be overcome with preservation steps. Another alternative would be a POC molecular testing strategy, but for STH, assays are not yet at the field-deployable stage due to the extensive pre-processing required. While molecular techniques require building up of expertise and thorough standardization, they have the added advantage of reducing variability due to varying levels of microscopic skills and duration of sample transport, and could also be used for detection of STH in the environment. After repeated rounds of MDA as part of the STH and LF control programs, and with improving sanitation, several endemic communities have transitioned to low-intensity infections. Due to the potential scope for elimination, there is a pressing need for highly sensitive molecular techniques for monitoring and evaluation of control programs and interventions.

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