

Research Paper

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Author for correspondence:

E.L. Agola,
E-mail: elelo@kemri.org

Development and evaluation of a loop-mediated isothermal amplification (LAMP) diagnostic test for detection of whipworm, *Trichuris trichiura*, in faecal samples

M.G. Ngari¹, I.N. Mwangi², M.P. Njoroge³, J. Kinyua¹, F.A. Osuna², B.M. Kimeu⁴, P.W. Okanya⁴ and E.L. Agola^{2,4} 

¹Department of Biochemistry, Jomo Kenyatta University of Agriculture and Technology, PO Box 62000, 00200 Nairobi, Kenya; ²Centre for Biotechnology Research and Development, Kenya Medical Research Institute (KEMRI), PO Box 54840, 00200 Nairobi, Kenya; ³Department of Health, PO Box 24, Kirinyaga County, Kerugoya, Kenya and ⁴Department of Biochemistry and Biotechnology, Technical University of Kenya, PO Box 52428, 00200 Nairobi, Kenya

Abstract

Whipworm infection or trichuriasis caused by *Trichuris trichiura* is of major public health concern in sub-Saharan Africa, particularly among pre-school and school-going children. It is among the neglected tropical diseases targeted for elimination through mass drug administration (MDA). One of the outcomes of MDA is a rapid decline in levels of infection intensity, making it difficult to monitor effectiveness of control measures using the conventional Kato–Katz procedure, which relies on the microscopic detection of parasite ova in faecal samples. In the present study, a loop-mediated isothermal amplification (LAMP) test was developed for the detection of *T. trichiura* infection in faecal samples. LAMP technology offers greater sensitivity and specificity than the microscopy-based tests. A set of four specific primers targeting the internal transcribed spacer 2 region of the ribosomal DNA were designed using Primer Explorer software. DNA was extracted from faecal samples using the alkaline lysis method (HotSHOT) and the LAMP reaction performed at 63°C for 1 h. The amplicons were visualized by both gel electrophoresis and with the naked eye following staining with SYBR green dye. Sensitivity and specificity tests were determined using the standard Kato–Katz diagnostic procedure as a reference test. The developed LAMP assay reliably detected *T. trichiura* DNA in faecal samples, with a specificity and sensitivity of 88% and 77%, respectively. No cross-reactivity was observed with several common helminth parasites. The developed LAMP assay is an appropriate diagnostic method for the detection of *T. trichiura* DNA in human faecal samples due to its simplicity, low cost, high sensitivity and specificity.

Introduction

Trichuris trichiura (family Trichuridae) nematode parasite or whipworm causes trichuriasis disease in humans and occurs worldwide, mostly in warm humid tropical countries. It is among the soil-transmitted helminth (STH) infections categorized as the neglected tropical diseases (NTDs) and targeted for elimination (WHO, 2010, 2017). *Trichuris trichiura* infects an estimated 450 million people worldwide, mostly school-age children (G/hiwot *et al.*, 2014). Chronic trichuriasis causes anaemia and poor nutrition status, especially in children, while heavy worm burdens can result in ulcerative colitis and rectal prolapse (Njiru *et al.*, 2016).

Diagnosis of trichuriasis, like other STH infections, relies on the microscopic detection of parasite eggs in faecal samples prepared using the Kato–Katz technique (Katz *et al.*, 1972) or the formol-ether concentration method (Allen & Ridley, 1970). While these procedures are relatively simple to perform and do not require expensive equipment, they have limited sensitivity and specificity, require expertise to make a correct diagnosis and can be tedious when processing large samples (George & McCulloch, 2011), leading to increased cases of misdiagnosis (Njiru *et al.*, 2016).

DNA-based diagnostic tests such as polymerase chain reaction (PCR) have proven to outperform microscopy in their reliability to detect gastro-intestinal parasitic infections (Phuphisut *et al.*, 2014; Acosta Soto *et al.*, 2017). However, despite its high-throughput screening potential, PCR requires costly equipment (Kaisar *et al.*, 2017), and, therefore, has not been able to replace microscopy in the diagnosis of STH infections in large-scale epidemiological surveys or in disease control programs.

The loop-mediated isothermal amplification (LAMP) technique, developed by Notomi *et al.* (2000), offers a viable alternative to microscopy or PCR as it amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions. This nucleic acid amplification technique requires only one enzyme (*Bst* DNA polymerase), and is able to amplify large amounts of DNA within 30–60 min by auto-strand displacement DNA synthesis (Notomi *et al.*, 2000). The advantages of this approach include the use of simple equipment, since the reactions take place at isothermal temperatures between 60°C and 65°C, and amplification products can easily be visualized by the naked eye after staining with SYBR green, a DNA-binding dye (Leal *et al.*, 2005). LAMP has emerged as a powerful tool for point-of-care diagnostics, and among the parasitic infections, LAMP diagnostic tests have been developed for the human malaria parasite *Plasmodium falciparum* (Han *et al.*, 2007), *Schistosoma haematobium* (Abbasi *et al.*, 2010), African trypanosomiasis (Kuboki *et al.*, 2003; Njiru *et al.*, 2008), hookworm *Necator americanus* (Mugambi *et al.*, 2015), *Ascaris lumbricoides* (Shiraho *et al.*, 2016) and *Schistosoma mansoni* (Mwangi *et al.*, 2018). Rapid and reliable diagnosis of trichuriasis is central to its control and elimination, as well as disease surveillance and monitoring. In the present study, we developed a LAMP test for the diagnosis of whipworm (*T. trichiura*) infections in human faecal samples.

Materials and methods

Study approval and ethical considerations

This study was approved by the Scientific and Ethical Review Unit (SERU) of the Kenya Medical Research Institute (KEMRI/SERU/CBRD/160/3397). Faecal samples used in this study were collected from school-going children in the endemic locality of Mwea, central Kenya. Faecal sample collection for diagnosis of trichuriasis and other common STH infection is a routine procedure and does not pose any risks or cause harm to participating children. The parents/guardians of the participating children gave written consent for their children to participate in the study, and children aged 12 years and above assented to participate. The samples of the study participants were coded and data stored in a password-protected personal computer. All the children found to be infected with *T. trichiura* or any other soil-transmitted intestinal helminth infection received treatment with a single dose of 400 mg albendazole and/or 40 mg/kg Paziquantel for Schistosomiasis, under the supervision of a clinician.

Parasite material

Faecal samples were collected from a total of 137 school-going children, aged 6–12 years from Mwea, Kirinyaga County, who were attending Mianya, Ngurubani and Mukou Primary Schools. Overall, the prevalence of *T. trichiura* infection among the children examined was 28%. Other faecal-based intestinal parasites present among the study children were hookworm (*N. americanus*), *S. mansoni* and *A. lumbricoides*.

Faecal sample collection and parasitological examination

Each participating child provided a single faecal sample in a capped plastic polypot, which were transported to the field laboratory in an ice box within 4 h of collection, and a double-slide

Kato–Katz sample was prepared on glass microscope slides according to the standard procedure and examined under a compound microscope at X400 magnification within 1 hr of sample preparation (Katz *et al.*, 1972). *Trichuris trichiura*-positive samples were used to isolate ova for DNA extraction used for optimizing the LAMP technique. Similarly, faecal samples that were positive for *N. americanus*, *S. mansoni* and *A. lumbricoides* provided parasite ova for DNA extraction for evaluation of the LAMP test for specificity. Negative samples were also collected and used for the test of agreement between the Kato–Katz technique and LAMP.

Isolation of parasite eggs from faecal samples

To optimize the technique, parasite ova were isolated from faecal samples to avoid contamination with other materials in faecal samples. This was done using the Modified Wisconsin Sugar Flotation Method (Goodman *et al.*, 2007). Briefly, Sheather's solution was prepared by adding 454 gm of table sugar to 355 ml of very hot water and stirred until it dissolved. This was then allowed to cool before use. Approximately 6 gm of faecal sample was then placed in a 3.5-inch diameter petri dish and 25 ml of the Sheather's solution was added. The mixture was stirred into a homogenate using a wooden tongue depressor and filtered through layered wet cheesecloth into a 50 ml centrifuge tube. The filtrate was centrifuged at 4000 rpm for 15 min, and the solution was left to stand for 1 h to allow the parasite eggs to float. The presence of parasite ova was confirmed under the microscope by placing a cover slip over the solution and transferring it onto a microscope slide. Approximately 5 ml of the supernatant was transferred into a 15 ml centrifuge tube, and two volumes of water added to dilute the sugar solution to reduce the specific gravity. This allowed the eggs to settle at the bottom after a 5 min spin in the centrifuge at 4000 rpm. The resulting pellet was re-suspended in 2 ml distilled water, vortexed and then transferred into 1.5 ml centrifuge tubes.

DNA extraction from isolated parasite eggs and from crude faecal samples

DNA extraction from isolated parasite eggs was performed using the modified alkaline lysis DNA extraction (HotSHOT) method (Truett *et al.*, 2000). Briefly, 10 µl of concentrated eggs was added to 30 µl of lysis buffer (sodium hydroxide, ethylenediaminetetraacetic acid and distilled water), followed by sample incubation at 95°C for 1 h. After incubation, the sample was rapidly cooled to 4°C followed by the addition of 30 µl of lysis buffer (Tris-hydrochloride and distilled water).

Genomic DNA was extracted from crude freshly collected human faecal samples using the QIAamp DNA Stool Mini Kit (QIAGEN, Massachusetts, USA; catalogue no. 51504) as per the manufacturer's instructions.

Primer design and selection

LAMP primers were designed, based on the internal transcribed spacer 2 (ITS-2) ribosomal DNA of *T. trichiura*, using Primer Explorer version 5.0 software (Fujitsu Ltd., Tokyo, Japan). This region was chosen due to its conserved nature and specificity to *T. trichiura*. Five generated primer sets were subjected to computation analysis by the basic local alignment search tool (BLAST; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to verify their suitability

and close relatedness to the *T. trichiura* from GenBank with sequence accession number GQ301555.1 (*T. trichiura* from *Homo sapiens* internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and ITS-2, partial sequence of a length of 1404 bp.). The one primer set (having FIP, BIP, F3 and B3) that generated reproducible results in optimization tests was chosen for subsequent experiments (table 1).

Optimization of the LAMP reaction conditions

The LAMP assay was optimized to determine the optimum incubation temperature, reaction time and primer ratios. Temperatures from 60°C to 65°C, reaction time from 30 min to 120 min and primer ratios of inner (FIP,BIP):outer (F3,B3) were tested. In this assay, optimum LAMP reaction conditions were found to be temperatures of 63°C for 60 min, and primer ratios were 4:2.5 (FIP,BIP:F3,B3), respectively.

LAMP reaction

LAMP reaction was performed as previously described (Notomi *et al.*, 2000). Initial optimization reactions were done using a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, Singapore), but all other subsequent experiments (sensitivity, specificity and evaluation) were performed on a water bath and the reaction stopped in boiling water to inactivate the *Bst* enzyme. The reaction mix had a final volume of 27 µl containing 1.48 pmoles (FIP and BIP) and 0.93 pmoles (F3 and B3), 1 µl of 8 U/µl of *Bst* DNA polymerase (New England BioLabs, Ipswich, Massachusetts, USA), 10× *Bst* buffer and 1 µl (10 ng/µl) target DNA. Amplification was carried out at 63°C for 60 min, and *Bst* DNA polymerase activity stopped by boiling in water for 10 min.

LAMP product detection

LAMP products were detected using two methods: (1) electrophoresis on a 2.0% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen by Thermo Fisher Scientific, Massachusetts, USA) and photographed on a gel documentation system (UVP VisiDoc-It Imaging System, CA, USA); and (2) staining the amplified products with SYBR green nucleic acid stain and visualization with the naked eye. Positive samples turned green in colour and the negative samples remaining orange in colour.

Evaluation of the LAMP assay for specificity and sensitivity in comparison with the Kato–Katz technique

The performance of the LAMP assay for detection of *T. trichiura* was evaluated for specificity and sensitivity against the microscopy-based Kato–Katz technique using 137 faecal samples collected from schoolchildren from Mwea.

To determine the specificity of the LAMP assay in detecting *T. trichiura* DNA, the LAMP primers designed for this purpose were tested in the assay using DNA extracted from the other common, faecal-based helminth parasites – namely, *A. lumbricoides*, hookworm (*N. americanus*) and *S. mansoni* – and the amplification products analysed by agarose gel electrophoresis and SYBR green staining alongside the amplified *T. trichiura* DNA.

To determine the analytical sensitivity of the assay, 1:10 serial dilutions of *T. trichiura* DNA were prepared from an initial solution at 98.6 ng/µl, and the DNA samples from each dilution were

Table 1. Primer sequences used for the amplification of *Trichuris trichiura* ova.

Primer	Sequence 5'→3'
F3	CGGAGAGCGGCTAACTCAG
B3	CGTCGTCGTCTCTAGTCG
FIP	AACGGTCGTTCCGCCGCTTTACAGTACGGAAGCTGCCCGA
BIP	ACCACCGAGCGACCACTGCCGCTGCCGTGATCTAGG

then amplified separately in the assay. The amplified products from each dilution were visualized on stained agarose gel after electrophoresis.

Data analysis

For the entire 137 human faecal samples, parasite detection was done using both the Kato–Katz procedure and the developed LAMP assay, and the results recorded as either positive or negative. The data were then analysed for sensitivity and specificity using the following formula developed by Cohen (1960):

$$K = \frac{OA - AC}{1 - AC}$$

where K is kappa value, OA is observed agreement and AC is agreement by chance.

Results

Primer design and selection for the LAMP reaction

Five primer sets were generated by Primer Explorer software version 5 (Fujitsu Ltd., Tokyo, Japan). After trying all primer combinations, one set of primers (table 1) gave consistent and reliable amplification. This was subsequently used in all the analysis and optimization of the technique.

LAMP reaction optimization

Primer concentration ratios

When ratios of different primer concentrations of inner (FIP, BIP):outer (F3, B3) between 1:1 and 8:10, respectively, were carried out, the most optimum ratio based on the brightness of the amplicon bands on the agarose gel after electrophoresis was observed to be 4:2.5 (fig. 1).

Reaction temperature

When different isothermal temperature conditions were tested between 60°C and 65°C, based on the brightness of DNA amplicons, the optimal temperature for reaction mix was determined to be 63°C (fig. 2)

Ability to detect single whipworm egg DNA

Whipworm parasite eggs were isolated from fresh faecal samples, counted and subjected to LAMP assay to determine the optimum number that can be successfully amplified; 1, 5, 10 and 15 eggs were tested, respectively. Amplification was possible even for a single egg (fig. 3).

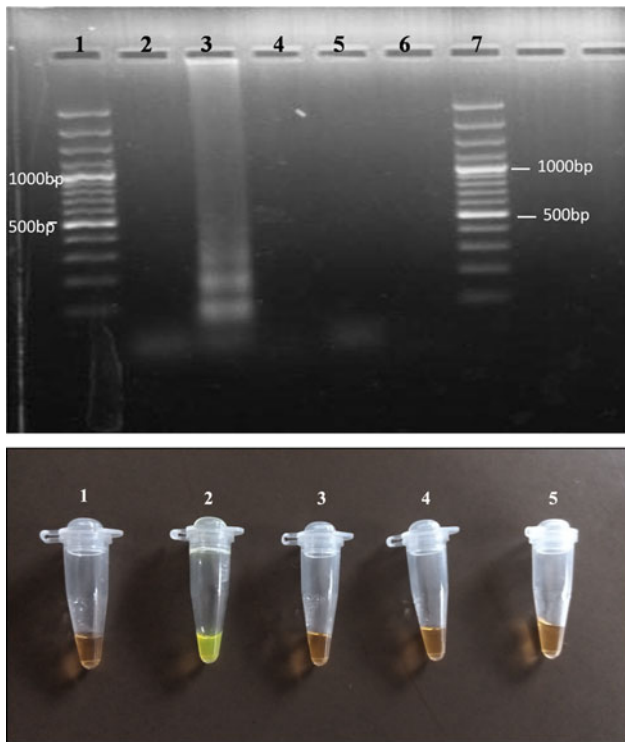


Fig. 1. (a) Agarose gel photo showing results of the LAMP assay when varying primer ratios. Lanes 2, 3, 4 and 5 represent ratios 1:1, 4:2.5, 6:5 and 8:10, respectively. Lane 6 is the negative control (reaction mix without DNA), and Lanes 1 and 7 are 100-bp molecular markers. (b) LAMP visual detection for colour change using the SYBR green dye. Tubes 1, 2, 3 and 4 represent primer ratios 1:1, 4:2.5, 6:5 and 8:10, respectively. Tube 5 is the negative control.

Specificity of the LAMP reaction

Only samples of *T. trichiura* were amplified with no cross-reactivity or amplification observed with closely related parasites, illustrating the high specificity of the developed LAMP assay, as in fig. 4.

Sensitivity of the LAMP reaction

When 1:10 serial dilutions of total genomic DNA from *T. trichiura* were used to test the sensitivity of the developed LAMP assay from an initial concentration of 98.6 ng/ μ l, the LAMP assay was able to amplify DNA concentration as low as 98.6×10^{-9} ng/ μ l (fig. 5).

Comparison of LAMP against Kato-Katz

When the performance of the developed LAMP technique was compared to the Kato-Katz technique, results displayed in a 2×2 contingency table, and sensitivity, specificity and predictive values determined, our results indicated that the LAMP technique had a sensitivity of 77% compared to that of Kato-Katz. The specificity was 88% (table 2).

$$\text{Observed agreement(OA)} = \frac{34 + 82}{137} = 0.85$$

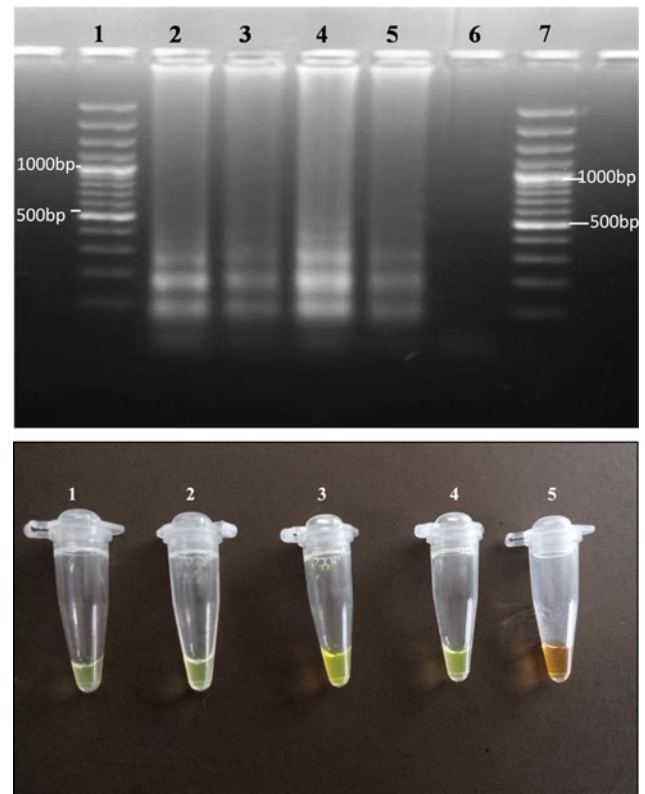


Fig. 2. (a) Agarose gel photo showing amplified fragments at temperatures in the range of 60–65°C for Lanes 2, 3, 4 and 5 representing 60°C, 62°C, 63°C and 65°C, respectively. Lane 6 is the negative control (reaction mix without DNA), Lanes 1 and 7 are 100-bp molecular markers. (b) Detection of amplicon by the naked eye based on colour change using the SYBR green dye. Tubes 1, 2, 3 and 4 represent amplification at temperatures of 60°C, 62°C, 63°C and 65°C, respectively. Tube 5 represents the negative control (reaction mix without DNA).

$$\begin{aligned} \text{Agreement by chance (AC)} &= \left(\frac{44}{137} \times \frac{45}{137} \right) + \left(\frac{93}{137} \times \frac{92}{137} \right) \\ &= 0.11 + 0.46 = 0.57 \end{aligned}$$

$$\text{Kappa } (k) = \frac{0.85 - 0.57}{1 - 0.57} = 0.66$$

showing substantial agreement.

$$\text{Sensitivity} = \frac{34}{44} \times 100 = 77.27\%$$

$$\text{Specificity} = \frac{82}{93} \times 100 = 88.17\%$$

This shows that 34 samples of 137 were positive for *T. trichiura* on both LAMP and Kato-Katz, 11 samples were negative on Kato-Katz, which were positive on LAMP, ten samples were positive on Kato-Katz, which were negative on LAMP, whereas both Kato-Katz and LAMP agreed 82 samples were negative.

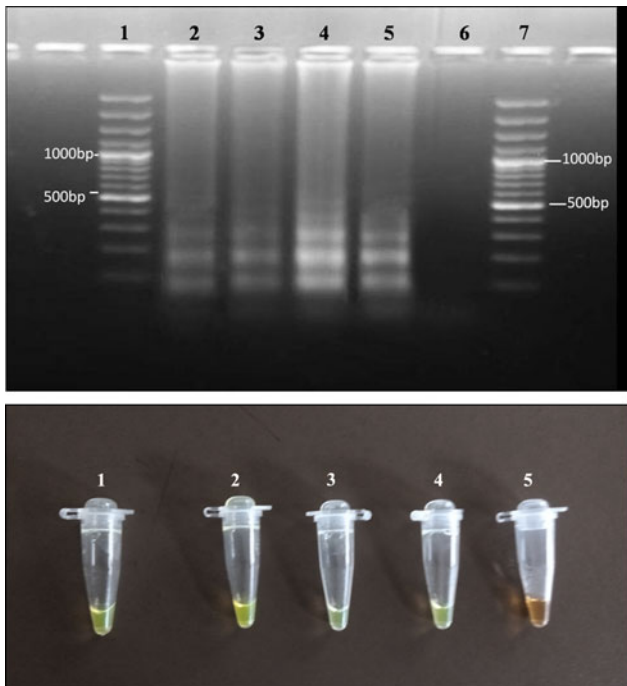


Fig. 3. (a) Agarose gel showing amplification of isolated egg(s). Lanes 2, 3, 4 and 5 are DNA amplification from 1, 5, 10 and 15 eggs, respectively. Lane 6 is the negative control (reaction mix without DNA) and Lanes 1 and 7 are 100-bp molecular markers. (b) Detection of amplicons using SYBR green dye. Tubes 1, 2, 3 and 4 represent amplifications of 1, 5, 10 and 15, respectively. Tube 5 is the negative control (reaction mix without DNA).

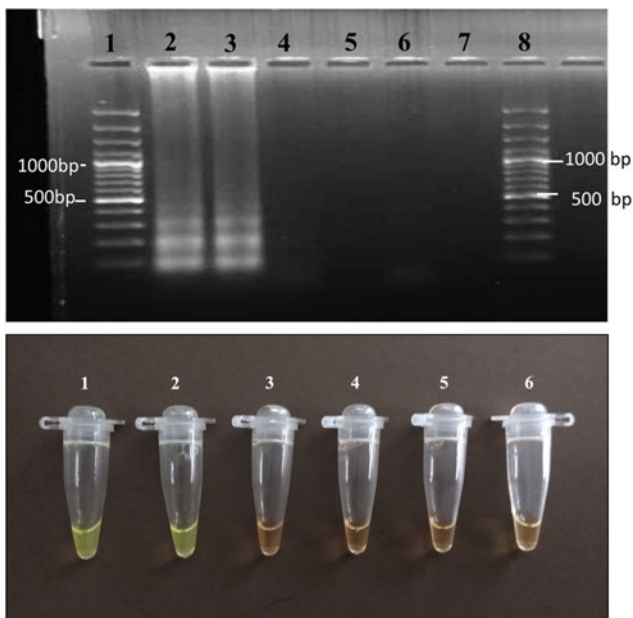


Fig. 4. (a) Agarose gel photo showing results for the specificity of the LAMP assay for *Trichuris trichiura*. Lanes 2 and 3 are *T. trichiura* DNA at 82.8 ng/μl and 99.2 ng/μl, respectively. Lane 4 is *Schistosoma mansoni* DNA at 98.6 ng/μl, Lane 5 is *Necator americanus* DNA at 90.6 ng/μl and Lane 6 is *Ascaris lumbricoides* at 50.2 ng/μl. Lane 7 is a negative control (master mix without DNA). Lanes 1 and 8 represent 100-bp molecular markers. (b) Detection using the SYBR green dye. Tubes 1 and 2 represent amplification of *T. trichiura* DNA, and 3, 4 and 5 are *S. mansoni* DNA, *N. americanus* DNA and *A. lumbricoides* DNA, respectively. Tube 6 represents a negative control (master mix without DNA).

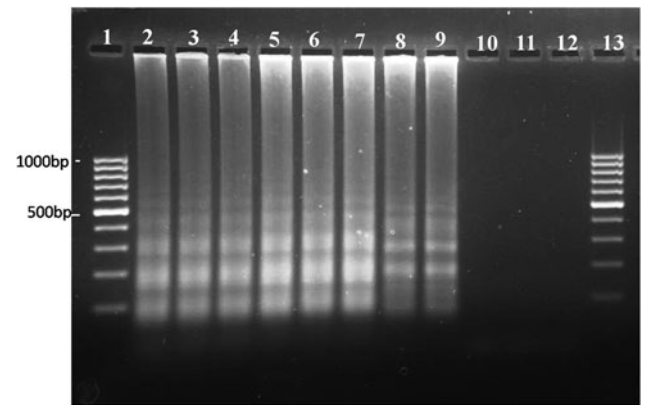


Fig. 5. Agarose gel photo of the LAMP assay amplified products of different concentrations of *Trichuris trichiura* DNA, following 1:10 serial dilutions, starting with a 98.6 ng/μl DNA concentration (Lane 2) to 98.6×10^{-9} ng/μl (Lane 11). Lane 12 is the negative control (master mix with no DNA), and Lanes 1 and 13 are 100-bp molecular markers.

Table 2. Contingency table comparing LAMP assay with the Kato-Katz technique.

		Kato-Katz		Total
		+	-	
LAMP	+	34	11	45
	-	10	82	92
Total		44	93	137

Discussion

In this paper, we present a LAMP method for the diagnosis of *T. trichiura* (whipworm) infection in faecal samples, with the ability to detect as low as 98.6×10^{-9} ng/μl parasite DNA, suggesting that it has the ability to detect very low levels of *T. trichiura* infections, which are typical of endemic areas where mass drug administration (MDA) is intensely used for control or elimination efforts against *T. trichiura* infections and other STH infections that often co-occur. Sensitive and accurate diagnostic tools for whipworm infections are critical for the detection of low-intensity infections such as those likely to be encountered where MDA is applied intensely or where low-level transmission of whipworm occurs (Webster *et al.*, 2014).

Although this assay was optimized using a thermocycler, all subsequent assays were done using a temperature controlled water bath to reliably amplify parasite DNA in the developed LAMP assay, suggesting its potential value for use in low-resource disease-endemic areas, where costly and sophisticated equipment may not be affordable for routine purposes or at the point of care (Amoah *et al.*, 2017).

In previous studies, it was shown that five of 12 (41.6%) parasitological negative cases by Kato-Katz gave positive results with PCR, indicating a clear case of misdiagnosis by Kato-Katz examination (Pontes *et al.*, 2003). Lower sensitivities of Kato-Katz (between 46.0 and 71.8) have also been observed (Knopp *et al.*, 2009). The low sensitivities of a single Kato-Katz thick smear (8.0% to 22.4%) have been attributed to low infection intensities and day-to-day variation in egg output (Booth *et al.*, 2003). Our findings indicate that LAMP has a sensitivity of 77% and a

specificity of 88%, with the ability of detecting single egg DNA, showing that it is more reliable compared to the Kato–Katz technique.

The generated primers were based on the ITS-2 of the ribosomal DNA, a region chosen due to the fact that it is conserved and has low mutation rates, and, also, is known to provide species-specific markers for differentiating even closely related species. In the present study, the primers designed were highly specific to *T. trichiura*, and had no cross-reactivity with other commonly occurring faecal-based helminth parasites such as *A. lumbricoides*, *S. mansoni* and hookworm (*N. americanus*). This is an important consideration since in many endemic localities, multiple helminth infections do frequently occur, and very often in one individual (Harris *et al.*, 2015).

LAMP has characteristics that give it an advantage over conventional PCR, making it ideal for use in a field set-up. For example, it does not require expensive equipment, as a simple water bath or heat block are used for isothermal amplification. Also, the simple detection system based on the staining of the amplicons with SYBR green nucleic acid stain, which is visualized by the naked eye, further simplifies the technique, giving it the potential to be used at the point of care in low-resource disease-endemic regions. The LAMP technique has already been used in rural laboratories in developing areas for the diagnosis of tuberculosis (Boehme *et al.*, 2007), trypanosomiasis (Njiru *et al.*, 2008) and asymptomatic malaria (Cook *et al.*, 2015).

While the LAMP diagnostic test has proved reliable and can be useful for field application, we do acknowledge the fact that it requires further improvements to make it applicable for routine use. For example, whether one is using a thermal cycler or a water bath, electricity is still required. This causes an addition of cost that makes it not applicable yet under field settings. Other improvements needed include a simple and precise DNA extraction method that will void the samples of polyphenols found in stool samples, which inhibits DNA polymerase. Also, pre-mixed and ready-to-use LAMP reagents are needed to minimize pipetting, which may result in contamination.

In conclusion, the developed LAMP has the advantage in specificity and sensitivity over the Kato–Katz method, and the assay described and developed in this study may constitute a turning point in the management of *T. trichiura*. The LAMP technique remains a ‘Gold Standard’ by which other isothermal DNA/RNA amplification methods are compared. It has the potential for use in the rapid diagnosis of *T. trichiura* infection, both in the laboratory and in a field set-up.

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Conflicts of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. The authors assert that all procedures

contributing to this work comply with the ethical standards of the relevant national and institutional guides on care and use of laboratory animals.

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