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A Loop-Mediated Isothermal Amplification (LAMP) Assay for *Strongyloides stercoralis* in Stool That Uses a Visual Detection Method with SYTO-82 Fluorescent Dye

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Abstract. An assay to detect *Strongyloides stercoralis* in stool specimens was developed using the loop-mediated isothermal amplification (LAMP) method. Primers were based on the 28S ribosomal subunit gene. The reaction conditions were optimized and SYTO-82 fluorescent dye was used to allow real-time and visual detection of the product. The product identity was confirmed with restriction enzyme digestion, cloning, and sequence analysis. The assay was specific when tested against DNA from bacteria, fungi and parasites, and 30 normal stool samples. Analytical sensitivity was to < 10 copies of target sequence in a plasmid and up to a 10^{-2} dilution of DNA extracted from a *Strongyloides ratti* larva spiked into stool. Sensitivity was increased when further dilutions were made in water, indicative of reduced reaction inhibition. Twenty-seven of 28 stool samples microscopy and polymerase chain reaction positive for *S. stercoralis* were positive with the LAMP method. On the basis of these findings, the assay warrants further clinical validation.

INTRODUCTION

Strongyloides stercoralis is a significant pathogenic nematode.[1] Conservative estimates of prevalence indicate 30–100 million people are infected worldwide.[1] Although strongyloidiasis is endemic to the tropics and subtropics, a history of travel in these areas is also a risk factor for chronic infection, which can persist for decades.[1,2] In severe infection, large numbers of larvae disseminate throughout the body.[1,2] This is associated with immune suppression and has a high mortality.[1,2] Maternal infection has been linked to low birth weight in infants.[3,4]

The morphological identification of *S. stercoralis* from stool has various applications for diagnosis based on symptoms, screening, and as a gold standard for the validation of serological tests.[1] It is particularly relevant where the sensitivity of serology is reduced, such as in immune suppressed states.[5] The most sensitive methods depend on the presence of live larvae.[1,6,7] These include agar plate culture and the Baermann technique, where larvae migrate through gauze and down a water-filled funnel.[1,6,7] Polymerase chain reaction (PCR)-based assays have also been developed for the detection of *S. stercoralis* in stool.[8–10] Nucleic acid tests have the advantage that *S. stercoralis* can be detected in specimens where the larvae have been killed, for example through freezing or in storage solutions such as ethanol.[8] This allows for the transportation of specimens that are not fresh and removes the risk of laboratory acquired strongyloidiasis.[8,11]

Molecular assays based on loop-mediated isothermal amplification (LAMP) of nucleic acid have been developed to diagnose a range of parasitic infections, including malaria, leishmaniasis, and cysticercosis.[12] The LAMP assays are relatively simple to perform, use stable reagents, have low infrastructure requirements, and have been applied in settings where resources are limited.[13–15] Cost-effective devices have been designed to improve the portability of the LAMP method for field applications.[16,17] The aim of this study was to apply the LAMP methodology to the diagnosis of strongyloidiasis, which would be suitable for use in areas with high prevalence. A LAMP assay for the detection of *S. stercoralis* was developed, using *Strongyloides ratti* as a laboratory model to determine analytical sensitivity and a preliminary evaluation was performed.

MATERIALS AND METHODS

Samples and DNA extraction methods. Laboratory cultured *S. ratti* L3-stage larvae were obtained using the Baermann technique.[18] The DNA was extracted from *S. ratti* larvae in water and larvae spiked into normal human stool using a spin column method, the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad CA), according to the manufacturer's instructions.[19] The DNA was extracted from a range of other parasites, fungi, and bacteria using standard methods (Table 1). These samples were either from pure cultures or, in the case of parasites, washed and concentrated organisms. Thirty stool specimens were collected from a low prevalence area (metropolitan Sydney) and were negative for *S. stercoralis* on microscopy, agar plate culture, and real-time PCR.[19] The DNA was extracted from these using the PowerSoil DNA Isolation Kit. These specimens were collected with the approval of the Human Research Ethics Committee, Sydney West Area Health Service. At a separate laboratory, DNA from 28 stool specimens, where *S. stercoralis* larvae were observed on microscopy, was extracted with a method that was modified by Verweij and others,[8] using QIAamp spin columns and Qiagen buffers (Qiagen, Hilden, Germany). These were obtained from collaborative projects in several different countries by the Department of Parasitology at the Leiden University Medical Center, with the approval of the Ethics Committee of Leiden University.

LAMP primers. Primers were designed to amplify *S. stercoralis* and *S. ratti* DNA. They were based on an archived partial sequence of the 28S rRNA gene for *S. stercoralis* (GenBank accession no. DQ14570.1), which was then aligned

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TABLE 1

Organism DNA tested with the *Strongyloides* (LAMP) assay for *Strongyloides stercoralis**

Human DNA	*Ancylostoma caninum*
Normal human stool	*Angiostrongylus cantonensis*
	Ascaris lumbricoides
Bacillus cereus	*Blastocystis hominis*
Bacteroides fragilis	*Cryptosporidium hominis*
Campylobacter jejuni	*Cryptosporidium parvum*
Citrobacter freundii	*Dientamoeba fragilis*
Clostridium difficile	*Enterobius vermicularis*
Enterococcus faecalis	*Fasciola hepatica*
Escherichia coli	*Giardia duodenalis*
Mycobacterium tuberculosis	*Necator americanus*
Proteus mirabilis	*Schistosoma japonicum*
Salmonella typhimurium	*Taenia saginata*
Shigella sonnei	*Trichuris trichiura*
Staphylococcus aureus	
Vibrio cholerae	*Aspergillus flavus*
Vibrio parahaemolyticus	*Aspergillus fumigatus*
Yersinia enterocolitica	*Candida albicans*
	Candida glabrata
	Candida krusei
	Candida parapsilosis
	Candida tropicalis
	Cryptococcus neoformans
	Penicillium chrysogenum

*All the results were negative. DNA concentrations ≥ 0.9 ng/μL.

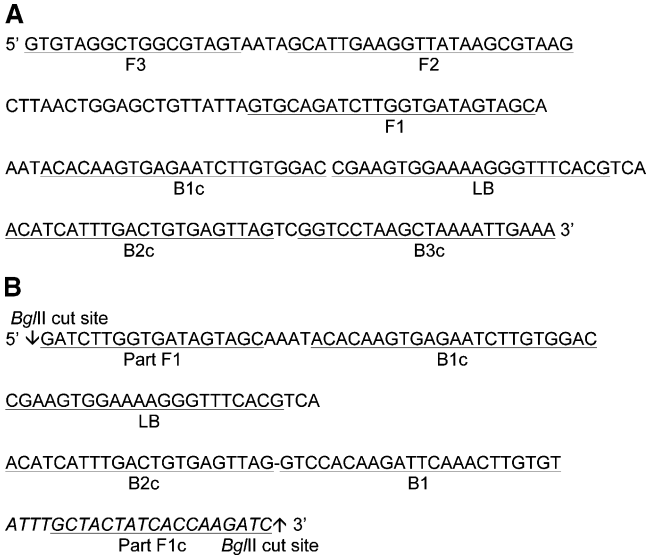

FIGURE 1. (**A**): Target sequence for loop-mediated isothermal amplification (LAMP) primers: forward outer primer (F3), backward inner primer (B3), forward inner composite primer (FIP; F1c-F2), backward inner composite primer (BIP; B1c-B2). c = reverse complement. (**B**): Sequence of the cloned LAMP product. An "alternately inverted repeat" consistent with a LAMP product digested by the *Bgl*II restriction enzyme. Depicted are F1 and F1c sequences flanking a "– B- +" sequence, characterized by Notomi and others.[20] Italics = reverse complement sequence.

with sequences from other *Strongyloides* spp. Specificity was checked by searching the GenBank *nt* database with the primer and target sequences for matches with other organisms. The Eiken Chemical Company (Tokyo, Japan) software "Primer Explorer V3" (http://primerexplorer.jp/e/) was used to design the primers. High-performance liquid chromatography (HPLC)-purified primers were as follows (5′–3′): forward outer primer (F3), GTGTAGGCTGGCG TAGT; backward outer primer (B3), TTTCAATTTTAGCT TAGGACC; forward inner composite primer (FIP; F1c-F2), GCTACTATCACCAAGATCTGCAC-GCATTGAAGGT TATAAGCGTAAG; backward inner composite primer (BIP; B1c-B2), ACACAAGTGAGAATCTTGTGGAC-CTAACTCACAGTCAAATGATGT; loop backward primer (LB), CGAAGTGGAAAAGGGTTTCACG. Figure 1 depicts where the primers bind to target DNA. The target sequence was compatible with the design of only one loop primer.

LAMP reaction solution and optimization. The LAMP reaction took place in a total volume of 25 μL. The reaction solution was modified from the protocol outlined by Tomita and others.[21] It included: thermopol buffer (1× dilution: 20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(NH_2)_2SO_4$, 0.1% Triton-X 100, 2 mM Mg_2SO_4; New England Biolabs, Ipswich, MA), 0.8 M betaine, dNTPs (1.4 mM each), and an additional 6 mM Mg_2SO_4. Primer amounts were 40 pmol FIP, 40 pmol BIP, 20 pmol LB, 5 pmol F3, and 5 pmol B3, with F3 and B3 amounts decreased from 10 pmol.[22] Bovine serum albumin (BSA) 0.1% (w/v) and polyvinylpyrrolidone (PVP) 1% (w/v) were added to counteract inhibition of the DNA polymerase.[23] Various methods of product detection were trialed based on calcein, hydroxynaphthol blue trisodium salt (HNB), SYTO-9 fluorescent dye (Life Technologies, Carlsbad, CA), SYBR-green dye (Life Technologies), and SYTO-82 fluorescent dye (Life Technologies).[21,24–27] After titration for reaction inhibition versus ease of visual detection, SYTO-82 was used in the final reaction solution at a concentration of 15 μM. The 500 μM SYTO-82 stock solution was made up in DMSO and stored frozen at –20°C. Purified water and 5 μL of sample DNA extract were added to the master mix to make up a volume of 24 μL. Before the addition of 8 units (1 μL) of *Bst* (large fragment) DNA polymerase (New England Biolabs), the reaction solution and DNA extract was heated to 95°C for 5 minutes and cooled to room temperature, to denature the template DNA and facilitate primer binding to the target sequences.[28] After pulse centrifugation, DNA polymerase was added to the tubes. The LAMP reaction occurred over 60 minutes by heating the solution to 60°C, a temperature that produced the shortest reaction time. The solution was then heated to 95°C for 3 minutes, to inactivate the DNA polymerase. The amplification of product was detected in real time using a Rotor-Gene 6000 (Corbett Research, Sydney, Australia; Figure 2), with the greatest fluorescence detected through the yellow channel (excitation 530 nm, detection 555 nm). At the completion of the reaction, a positive result was also detectable against a dark background with the naked eye under normal white light, or with a UVA fluorescent black "party" light (Nelson Industries, Hoxton Park, Australia; Figure 3). Agarose gel electrophoresis was used as a gold standard method of product detection.

Confirmation of product identity. To confirm the identity of the product, the restriction enzyme, *Bgl*II (New England BioLabs) was used according to manufacturer's instructions to digest 1 μL of the product following the LAMP reaction. This was then visualized using 2% agarose gel electrophoresis. The DNA from a prominent gel band was extracted and cloned by ligation into the plasmid vector, pBC SK+ (Stratagene, La Jolla, CA), that had been digested with *Bam*HI (New England BioLabs), and transformed into chemically

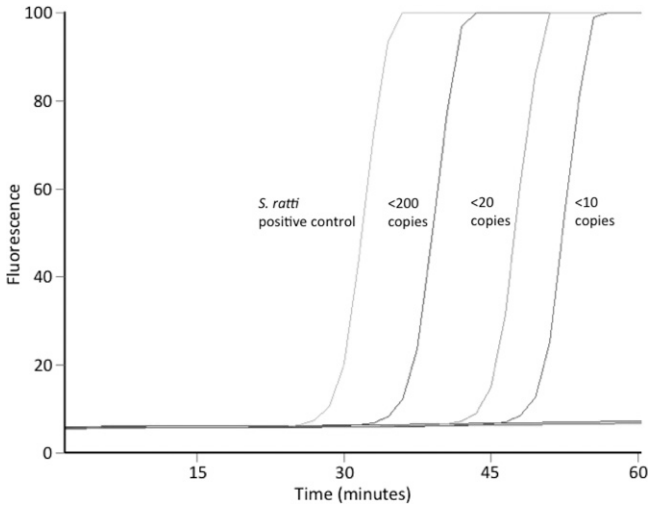

FIGURE 2. Rotor-Gene 6000 real-time detection curves for serial dilutions of the loop-mediated isothermal amplification (LAMP) target inserted into a plasmid, using SYTO-82 fluorescent dye: *Strongyloides ratti* positive control; < 200 copies of plasmid; < 20 copies of plasmid; < 10 copies of plasmid; horizontal lines, H_2O (no template) control, and dilution to < 2 copies of plasmid. Data acquisition: yellow channel (excitation 530 nm; detection 555 nm).

competent *Escherichia coli* DH5α, with *lacZ*-based *blue-white* selection. Insert regions were amplified and sequenced using primers M13F and M13R.

An alternative method to confirm product identity was also examined. This was based on PCR of the LAMP product using primers F2 and B2.[29] HotStarTaq DNA polymerase and PCR buffer (Qiagen) were used according to instructions from the manufacturer.

Analytical specificity. The DNA extracted from the bacteria, fungi, and parasites listed in Table 1 were tested. The absorbance at 260 nm was used to determine the presence of extracted DNA. In addition, DNA extracted from the 30 direct smear, agar plate culture, and real-time PCR negative stool specimens from a low prevalence area was tested. Non-template controls were used with every batch of reaction solution to monitor for cross-contamination or template free amplification.[30,31]

Analytical sensitivity. PCR primers for either side of the LAMP target region were designed: forward GCAGCC TTGAAAATGGATGG; reverse CTGTTGCGGATATGG GTACG. The PCR was performed using HotStarTaq DNA polymerase and PCR buffer to amplify the LAMP target sequence from cultured and purified *S. ratti* larvae. The product was then cloned, by ligation into the plasmid vector, pGEM-T Easy (Promega, Madison, WI), using the manufacturer's kit and transformed into chemically competent *E. coli* DH5α with *lacZ*-based *blue-white* selection. To confirm the presence of the LAMP target, the inserted region was amplified and sequenced using primers M13F and M13R. The Purelink Quick Plasmid Miniprep kit (Life Technologies) was used to purify the plasmid and the absorbance at 260 nm was used to measure the DNA concentration. Plasmids purified from two separate colonies were then serially diluted in Tris-EDTA buffer to determine a limit of detection for the LAMP assay.

Single live *S. ratti* larvae were aspirated using a 10 μL pipette with 1 μL of water. The larvae were then placed into 2 mL tubes containing 50 μL of water. The presence of one larva per tube was confirmed by direct visualization through a dissection microscope. This was added to 250 μL of human stool that was negative on direct smear, agar plate culture, real-time PCR, and previous testing with the LAMP assay. The DNA was extracted using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories), according to the instructions from the manufacturer, and eluted in 100 μL of Tris-EDTA buffer. Portions of the same stool sample, without the addition of *S. ratti*, were concurrently extracted, to provide a diluent. This was used to make serial dilutions using five replicates of the *S. ratti* spiked samples, to determine a limit of detection. For one of the replicates, the dilution that was the limit of detection and the first negative dilution in the series were further diluted to 10^{-1} and 10^{-2} with water and the LAMP assay performed.

Amplification and detection of *Strongyloides stercoralis* DNA. The LAMP assay was used to test 28 stool samples that were positive for *S. stercoralis* on microscopy and PCR, according to the method of Verweij and others.[8] Negative samples were then diluted 10^{-1} times with water and retested.

RESULTS

LAMP reaction optimization. The addition of the loop primer and the reduced amounts of primers F3 and B3 shortened the reaction time and the optimum temperature range was from 59 to 60°C.[22,32] Preheating of the reaction solution before adding the DNA polymerase also shortened the reaction time and resulted in a 10-fold increase in assay sensitivity on serial dilutions.

Product was detected using calcein, SYBR-green, SYTO-9, and SYTO-82.[21,25–27] The addition of PVP to the master mix changed the color of the HNB in the master mix and made positive and negative reactions difficult to differentiate.[24] Calcein and SYTO-9 could be added to the master mix before the reaction. Calcein allowed the visual detection of product, but the fluorescence saturated the Rotor-Gene detector. The SYTO-9 allowed real-time detection without visible fluorescence and higher concentrations inhibited the reaction before visual detection. Positive tubes fluoresced after the addition of SYBR-green following the LAMP reaction. The SYTO-82 was added before the reaction and allowed detection in real-time with the Rotor-gene and produced fluorescence that was detectable with the naked eye (Figures 2 and 3). At a 15 μM concentration of SYTO-82, there was a 3-minute increase in the time to detect product compared with a 5 μM concentration. However, with a 15 μM concentration visible fluorescence was more intense and sensitivity was not decreased. There was 100% concordance with the results of agarose gel electrophoresis.

Confirmation of product identity. The cloned fragment of the LAMP product (Figure 1) was consistent with the sequence configuration outlined by Notomi and others.[20] The F1 and F1c sequences flank a "– B- +" sequence. The incorporated reverse complement sequence was characteristic of a LAMP product. The F2 and B2 primers were used for PCR amplification of a portion of the LAMP product.[29] However, in this case, the PCR amplified LAMP product sequence was identical to the original *Strongyloides* sequence targeted by the LAMP primers, and not particular to the LAMP reaction.

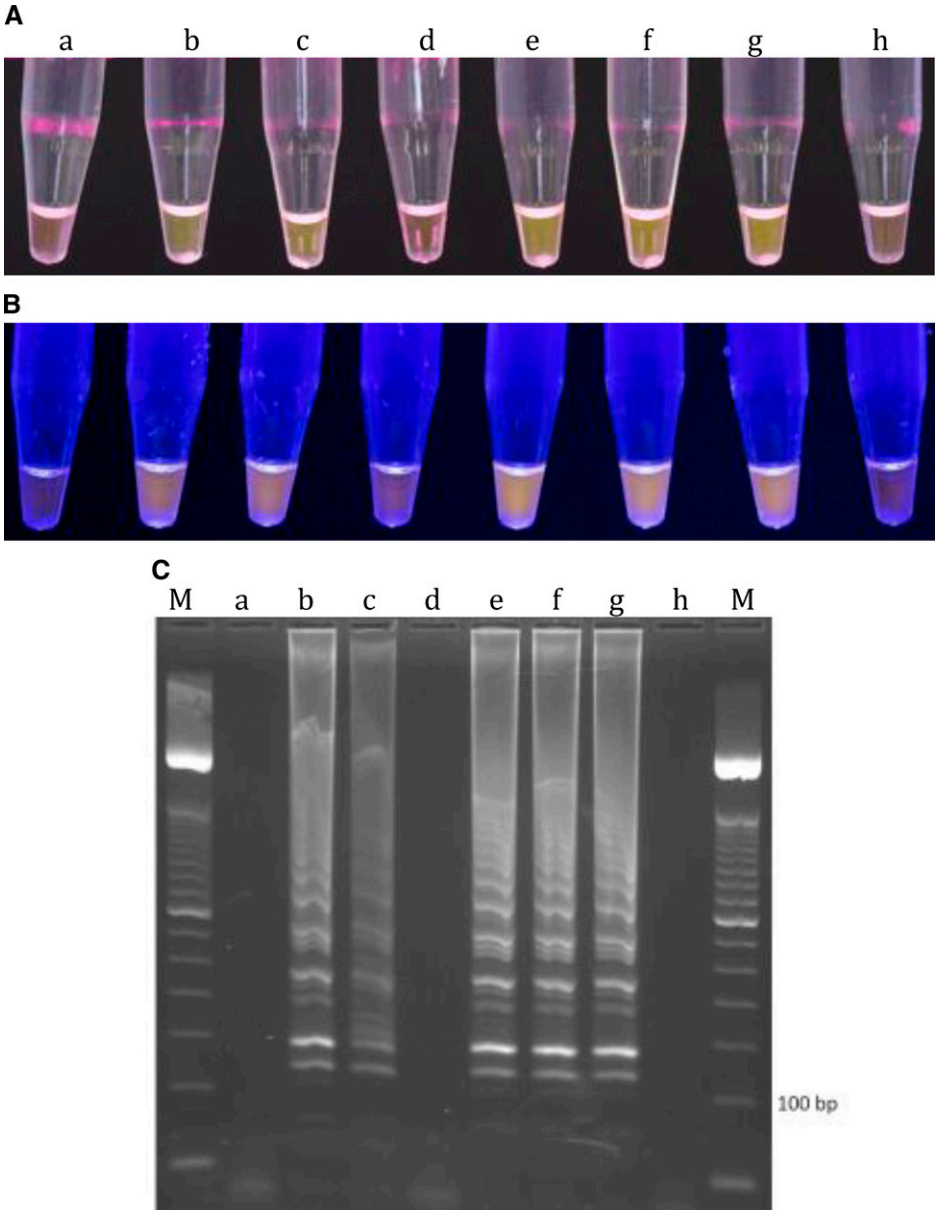

FIGURE 3. Tubes following the loop-mediated isothermal amplification (LAMP) reaction with SYTO-82 fluorescent dye visualized under (**A**) white fluorescent light and (**B**) a UVA light. (**C**) LAMP products visualized with 2% agarose gel electrophoresis. (a) H_2O non-template control; (b) *Strongyloides stercoralis* specimen; (c) target plasmid < 10 copies; (d) non-spiked stool extract (diluent for f to h); (e) neat 1× *Strongyloides ratti* spiked into stool; (f) 10^{-1} dilution 1× *S. ratti* spiked into stool; (g) 10^{-2} dilution 1× *S. ratti* spiked into stool; (h) 10^{-3} dilution 1× *S. ratti* spiked into stool; (M) 50 bp molecular weight marker. (a), (d), (h): negative reactions. (b), (c), (e), (f), (g): positive reaction.

Analytical specificity and sensitivity. The DNA from bacteria, fungi, and parasites was negative in the LAMP assay (Table 1). The thirty negative control stool samples from a low prevalence area were also negative. No template free amplification was shown with the use of template free controls.

The limit of detection of the assay for both plasmids was < 10 copies of DNA (Figure 2). The limit of detection based on single *S. ratti* larvae spiked into stool and diluted in stool extract was 10^{-2} for 4 replicates and 10^{-1} for 1 replicate (Figure 3). Samples that were diluted in stool extract to 10^{-2} (limit of detection) and 10^{-3} (first negative) and were subsequently diluted in water were positive at a 10^{-1} dilution and negative at a 10^{-2} dilution.

Detection of *Strongyloides stercoralis* DNA. On initial testing of the 28 samples that were microscopy and PCR positive for *S. stercoralis*, 26 were positive with PCR cycle threshold values (C_t) ranging from 20.23 to 38.02. To investigate for the presence of reaction inhibition, the two negative samples were then diluted 10^{-1} in water and retested. One was positive (PCR C_t 37.94) and the other remained negative (PCR C_t 38.44).

DISCUSSION

We have developed a simple, isothermal nucleic acid amplification assay, using LAMP methodology, for the detection of

S. stercoralis in stool. In addition, some modifications were applied to the standard LAMP protocol.[21]

The preheating of the reaction mixture and sample DNA to 95°C, before the addition of the heat labile DNA polymerase, was found to increase sensitivity.[33] Another approach is to heat and denature the sample DNA before its addition to the reaction solution with enzyme.[21] However, this method requires the denatured sample DNA to be kept on ice before the LAMP reaction.

The SYTO-82 fluorescent dye was used as the method of choice for the detection of reaction product because it could be added before the LAMP reaction without significant inhibition, binds directly to DNA, and allowed for real-time and visual detection. Although there was some increase in the time to product detection with real-time monitoring using a concentration of 15 mM, visual detection was improved. If visual detection was not necessary, lower concentrations of SYTO-82 may be used for real-time detection.

Because it was not feasible to routinely sequence the LAMP reaction products and a hybridization probe was not used in this protocol, the LAMP primers needed to be specific. Stool may contain a broad range of commensal and pathogenic organisms. Although a basic local alignment search tool (BLAST) search of GenBank and other databases can identify cross-reactivity *in silico*, not all sequences are available and *in vitro* testing was also required. Primers used for isothermal amplification may also exhibit template free amplification, which can lead to false positive results.[30,31] If this occurs, primer redesign is recommended (http://loopamp.eiken.co.jp/e/lamp/primer.html).

Methods for the diagnosis of *S. stercoralis* in stool are ideally highly sensitive because of the variable larval output in chronic infection.[34] The LAMP reaction using serial dilutions of plasmid showed a high sensitivity comparable to other LAMP assays.[28,29,31] However, the sensitivity of the assay using stool specimens will also be determined by the capacity of the extraction method to retain purified DNA, inactivate DNases, and remove polymerase inhibitors, such as bile salts and plant-based polysaccharides.[35,36] In *S. stercoralis* infection where low numbers of larvae may have an uneven distribution in the stool, the amount of stool used in the extraction method may also be significant.[19] Where *S. ratti* was spiked into stool as a model for *S. stercoralis* infection, the analytical sensitivity of the LAMP assay was comparable to the results for PCR (10^{-2} dilution of 1 spiked larva), when an estimate of larval number was used for spiking and dilutions were made in water, rather than stool extract.[19] The LAMP assay sensitivity was increased on further dilution of the *S. ratti* spiked extracts in water, indicating that inhibitors remained in the extracted samples.

Strongyloides PCR has shown clinical sensitivities from 34% to 83% and clinical specificities from 92% to 99%, using freshly cultured or Baermanized stool specimens as a gold standard.[8,19,37] Some of this variation in sensitivity may be caused by the use of different extraction techniques and the period of time stool specimens were stored frozen or in ethanol, before DNA extraction.[8,19,37] The comparison of the LAMP assay to PCR performed on extracts from stool that were microscopy positive for *S. stercoralis* showed concordance for samples with a wide range of PCR C_t values. The only sample that was negative using the LAMP assay had a high C_t value (> 38) with real-time PCR. This difference in sensitivity at a low concentration of template DNA may have been related to factors such as the presence of polymerase inhibitors, DNA binding to plastic ware, and variation in the amount of pipetted template, caused by stochastic distribution.[38]

Limitations of the assay include the variable larval output with potentially low numbers in stool specimens, the practicalities of obtaining stool specimens from patients, and the need for a laboratory to perform DNA extraction from stool and to have precautions in place to prevent cross-contamination with DNA, which can lead to false positive results.[34] However, these constraints are shared by conventional tests for *S. stercoralis* in the stool or nucleic acid tests in general. Before implementation, this assay requires additional validation using clinical specimens, where results can be compared with existing diagnostic methods with the calculation of clinical sensitivity, clinical specificity, and predictive values. In a clinical validation study, the use of neat and diluted samples may improve sensitivity, depending on the presence of polymerase inhibitors following DNA extraction.

A LAMP assay with a versatile detection method has been developed for the diagnosis of *S. stercoralis* in stool, a preliminary evaluation has been performed, and clinical validation is warranted. The visual detection of positive results and the use of basic heating devices would reduce the infrastructure requirements in areas endemic for strongyloidiasis. With the use of simplified or miniaturized extraction methods, the test would also be easier to perform in remote areas.

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