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...
and others.\textsuperscript{21} It included: thermopol buffer (1 solution was modified from the protocol outlined by Tomita reaction took place in a total volume of 25

Various methods of product detection, SYTO-82 was used in the final reaction solution at
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**LAMP DETECTION OF STRONGYLOIDES STERCORALIS**

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<th>Organism DNA tested with the Strongyloides (LAMP) assay for Strongyloides stercoralis*</th>
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<th>Bacillus cereus</th>
<th>Bacteroides fragilis</th>
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*All the results were negative. DNA concentrations ≥ 0.9 ng/µL.

with sequences from other Strongyloides spp. Specificity was checked by searching the GenBank *n* 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), TTCTAATTTTACTG TAGAAGCTGG; forward inner composite primer (FIP; F1c-F2), GTCACTATACCAAGATCTGAC-GCATTTAGGAAGTT TATAAGGGTGAAG; backward inner composite primer (BIP; B1c-B2), ACAACAAGTGAGAATTCTTGTGAGC C-CTAACTACAGTCAATAGTGT; loop backward primer (LB), CGAAAGTGAAAGGGTTTTCAGTCA. 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.\textsuperscript{21} It included: thermopol buffer (1 x dilution: 20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1% Triton-X 100, 2 mM MgSO₄, New England Biolabs, Ipswich, MA), 0.8 M betaine, dNTPs (1.4 mM each), and an additional 6 mM MgSO₄. 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.\textsuperscript{22} Bovine serum albumin (BSA) 0.1% (w/v) and polyvinylpyrrolidone (PVP) 1% (w/v) were added to counteract inhibition of the DNA polymerase.\textsuperscript{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).\textsuperscript{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.\textsuperscript{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 UV A 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, BglII (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 BamHI (New England Biolabs), and transformed into chemically
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. Negative samples were then diluted 10^3 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. 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. 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. 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. 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. 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.
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 \(10^{-3}\) 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 \(10^{-1}\) (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 (Ct) 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 Ct 37.94) and the other remained negative (PCR Ct 38.44).

DISCUSSION

We have developed a simple, isothermal nucleic acid amplification assay, using LAMP methodology, for the detection of *Strongyloides stercoralis* DNA.
S. stercoralis in stool. In addition, some modifications were applied to the standard LAMP protocol.\textsuperscript{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.\textsuperscript{33} Another approach is to heat and denature the sample DNA before its addition to the reaction solution with enzyme.\textsuperscript{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 \textit{in silico}, not all sequences are available and \textit{in vitro} testing was also required. Primers used for isothermal amplification may also exhibit template free amplification, which can lead to false positive results.\textsuperscript{30,31} If this occurs, primer redesign is recommended (http://loopamp.eiken.co.jp/e/lamp/primer.html).

Methods for the diagnosis of \textit{S. stercoralis} in stool are ideally highly sensitive because of the variable larval output in chronic infection.\textsuperscript{34} The LAMP reaction using serial dilutions of plasmid showed a high sensitivity comparable to other LAMP assays.\textsuperscript{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.\textsuperscript{35,36} In \textit{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.\textsuperscript{19} Where \textit{S. ratti} was spiked into stool as a model for \textit{S. stercoralis} infection, the analytical sensitivity of the LAMP assay was comparable to the results for PCR (10\textsuperscript{-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.\textsuperscript{19} The LAMP assay sensitivity was increased on further dilution of the \textit{S. ratti} spiked extracts in water, indicating that inhibitors remained in the extracted samples.

\textit{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.\textsuperscript{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.\textsuperscript{1,19,37} The comparison of the LAMP assay to PCR performed on extracts from stool that were microscopy positive for \textit{S. stercoralis} showed concordance for samples with a wide range of PCR C\textsubscript{T} values. The only sample that was negative using the LAMP assay had a high C\textsubscript{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.\textsuperscript{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.\textsuperscript{34} However, these constraints are shared by conventional tests for \textit{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 \textit{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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LAMP DETECTION OF STRONGYOLOIDES STERCORALIS


