Development of a Highly Sensitive Loop-Mediated Isothermal Amplification (LAMP) Method for the Detection of *Loa loa*

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Abstract

The filarial parasite *Loa loa*, the causative agent of loiasis, is endemic in Central and Western Africa infecting 3–13 million people. *L. loa* has been associated with fatal encephalopathic reactions in high *Loa*-infected individuals receiving ivermectin during mass drug administration programs for the control of onchocerciasis and lymphatic filariasis. In endemic areas, the only diagnostic method routinely used is the microscopic examination of mid-day blood samples by thick blood film. Improved methods for detection of *L. loa* are needed in endemic regions with limited resources, where delayed diagnosis results in high mortality. We have investigated the use of a loop-mediated isothermal amplification (LAMP) assay to facilitate rapid, inexpensive, molecular diagnosis of loiasis. Primers for LAMP were designed from a species-specific repetitive DNA sequence from *L. loa* retrieved from GenBank. Genomic DNA of a *L. loa* adult worm was used to optimize the LAMP conditions using a thermocycler or a conventional heating block. Amplification of DNA in the LAMP mixture was visually inspected for turbidity as well as addition of fluorescent dye. LAMP specificity was evaluated using DNA from other parasites; sensitivity was evaluated using DNA from *L. loa* 10-fold serially diluted. Simulated human blood samples spiked with DNA from *L. loa* were also tested for sensitivity. Upon addition of fluorescent dye, all positive reactions turned green while the negative controls remained orange under ambient light. After electrophoresis on agarose gels, a ladder of multiple bands of different sizes could be observed in positive samples. The detection limit of the assay was found to be as little as 0.5 ag of *L. loa* genomic DNA when using a heating block. We have designed, for the first time, a highly sensitive LAMP assay for the detection of *L. loa* which is potentially adaptable for field diagnosis and disease surveillance in loiasis-endemic areas.

Introduction

Loiasis is a neglected tropical disease caused by the nematode *Loa loa*, an endemic filarial parasite transmitted by Tabanid flies of the genus *Chrysops* in the forest and savannah regions of Central and Western Africa infecting between 3 and 13 million people. Although typical clinical presentation of loiasis includes oedema (Calabar swellings), migration of the adult worm through the sub-conjunctiva ("African eyeworm") and pruritus, infected individuals may exhibit a range of symptoms. On the other hand, indigenous inhabitans of endemic areas are typically clinically asymptomatic even in the setting of high parasite burden [1].

In recent years, *L. loa* has come increasingly to light as it has been associated with severe adverse reactions (including fatal encephalopathies) in high *Loa*-infected individuals receiving ivermectin during mass drug administration programs for the control of onchocerciasis and lymphatic filariasis. This had a negative impact on control of these two diseases in several areas of

co-endemicity with loiasis [2,3]. A greatest risk for *Loa*-related post-ivermectin encephalopathy has been reported to appear with microfilaremia above 8,000 organism/mL [4,5]. In this context, it is necessary the identification of individuals with high-level *L. loa* microfilaremia in order to prevent the interruption of mass drug administration programs in potentially loiasis endemic areas where ivermectin treatment was initially planned.

In endemic areas, the only diagnostic method routinely used is the microscopic examination of mid-day blood samples by thick blood film. This method requires expert training in parasite morphology and requires a great effort to process large numbers of samples so, in practice, is not suitable for large-scale surveys. Serological assays are an alternative, but suffer from poor specificity [6] and cannot discern between active and prior infection or quantify microfilaremia [7,8,9]. Regarding the molecular methods, in recent years both conventional [10,11,12,13,14] and real-time PCR assays [15] have been

developed for *L. loa* DNA detection. While these studies have demonstrated that PCR-based assays provided reliable, specific and sensitive tools, they are not generally available for use in endemic areas because highly skilled personnel and precision thermocyclers are needed. Therefore, the development of cost-effective, simple and rapid detection methods is still needed for the diagnosis of loiasis.

An alternative to PCR is a technique named loop-mediated isothermal amplification (LAMP). This assay is a one-step amplification reaction that amplifies a target DNA with high specificity, efficiency and rapidly under isothermal conditions [16]. LAMP employs a DNA polymerase (*Bst* polymerase) with strand-displacement activity, along with two inner primers (FIP, BIP) an outer primers (F3, B3) which recognize six separate regions within a target DNA. Additionally, the LAMP assay does not require an expensive thermocycler and allows simple visual detection of products [17,18]. It has been applied successfully to detect several pathogenic parasites, including filarial parasites, such as *Dirofilaria immitis* [19], *Brugia* spp. [20] and *Wuchereria bancrofti* [21].

The objective of this work was to design, for the first time, a LAMP-based approach for the highly sensitive detection of *L. loa* DNA potentially adaptable for field diagnosis of loiasis.

Materials and Methods

Ethics statement

The *Loa loa* adult worm used as source of DNA in our study was obtained by surgical removal from the eye of a Gabonese immigrant patient infected with the parasite as part of public health diagnostic activities at Tropical Medicine Consultation of University Hospital of Salamanca, Spain (Protocol no. 22.832). Then, sample was preserved in 70% ethanol. Patient was given detailed explanations about the aims, procedures and possible benefit of the study. Written informed consent was obtained from patient and sample was coded. Dr. Miguel Cordero (Specialist Head of Tropical Medicine Consultation) had contact with the patient and also had access to the patient's records.

The human blood samples used in this study were obtained from healthy blood donors (laboratory staff); participation was voluntary and all participants were given detailed explanations about the aims, procedures and possible benefit of the study. Written informed consent was obtained from all subjects and samples were coded and treated anonymously.

DNA extraction

Parasites. *Loa loa* genomic DNA was extracted from an adult worm preserved in 70% ethanol using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The concentration of *L. loa* DNA adult worm was measured three times by spectrophotometry using a Nanodrop ND-100 spectrophotometer (Nanodrop Technologies) to obtain an average concentration and then diluted with ultrapure water to a final concentration of 2.5 ng/μL. Subsequently, a 10-fold serial dilution was prepared ranging from 1×10^{-1} to 1×10^{-13} ng/μL and stored at $-20°C$ until use. Several helminths, including four nematodes namely *Mansonella perstans*, *Brugia pahangi*, *Strongyloides venezuelensis* and *Anisakis simplex*, seven trematodes namely *Schistosoma mansoni*, *S. haematobium*, *S. intercalatum*, *S. bovis*, *Fasciola hepatica*, *Echinostoma caproni* and *Dicrocoelium dendriticum*, and three cestodes namely *Hymenolepis diminuta*, *Taenia taeniformis* and *Echinococcus granulosus* were included as heterogeneous control samples for assessing the specificity of LAMP assays. All these parasites materials were preserved in 70% ethanol and kept at $-20°C$ until the extraction of DNA. Then, DNA was extracted and measured by the same

methods as described for *L. loa* adult worm. In order to look for protein contaminations a common purity check by measuring the A_{260}/A_{280} ratio was made for all samples.

Blood samples. Whole blood was taken from healthy staff donors, divided into aliquots of 200 μL and then artificially contaminated with genomic DNA from *L. loa* 10-fold serially diluted as prepared above. Then, DNA was extracted using NucleoSpin Blood kit (Macherey-Nagel, Germany) following the manufacturer's instructions.

Loa loa specific LAMP oligonucleotide design

An exclusive 839 base pair (bp) species-specific repetitive DNA sequence from *L. loa* was retrieved from GenBank (Accesion No. M34259.1) [10] and was used for the design of specific primers with Primer Explorer V4 software (Eiken Chemical Co., Ltd., Japan; http://primerexplorer.jp/e/). The primers were designed and selected based on the criteria described in "A Guide to LAMP primer designing" (http://primerexplorer.jp/e/v4_manual/index.html). Specific LAMP primer set selected consisting of primers F3, B3, FIP (F1c+F2) and BIP (B1c+B2) are shown in table 1. All of the primers were of HPLC grade (TIB-MOLBIOL, Germany).

LAMP F3 and B3 primer specificity tested by PCR. The outer LAMP primer pair, designated F3 and B3 (Table 1), was initially tested for *L. loa* specificity by PCR to verify whether the correct target was amplified. An amplicon of 189 bp was expected for *L. loa*. The PCR assay was conducted in 25 μL reaction mixture containing 2.5 μL of 10x buffer, 1.5 μL of 25 mmol/L MgCl$_2$, 2.5 μL of 2.5 mmol/L dNTPs, 0.5 μL of 100 pmol/L F3 and B3, 2 U *Taq*-polymerase and 2 μL of DNA template. Initial denaturation was conducted at 94°C for 1 min, followed by a touchdown program for 15 cycles with successive annealing temperature decrements of 1.0°C every 2 cycles. For these 2 cycles, the reaction was denatured at 94°C for 20 s followed by annealing at 59°C–57°C for 20 s and polymerization at 72°C for 30 s. The subsequent 15 cycles of amplification were similar, except that the annealing temperature was 56°C. The final extension was performed at 72°C for 10 min.

The sensitivity of the PCR was also assayed to establish the detection limit of *L. loa* DNA with 10-fold serial dilutions prepared as mentioned above. The assay was performed with 2 μL of the diluted template in each case. Negative (no DNA template) controls were included. The PCR products (10 μL of each) were subjected to 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light.

LAMP assay

The LAMP assay was performed using the *Loopamp DNA amplification Kit* (Eiken Chemicals Co., Tokyo, Japan) following manufacturer's instructions. Briefly, the reaction was carried out with a total of 25 μL reaction mixture containing 12.5 μL of 2x Reaction Mix, 40 pmol of each FIP and BIP primers, 5 pmol of each F3 and B3 primers, 1 μL of *Bst*-DNA polymerase, along with 2 μL of DNA template. To establish the standard protocol for the LAMP method, the reaction mixture was incubated in a conventional heating block (K Dry-Bath) or in a thermocycler (Mastercycler Gradient-96well; Eppendorf) at a range of temperatures (61, 63 and 65°C) for 60 min to optimize the reaction conditions and then heated at 80°C for 5 min to terminate the reaction. The optimal temperature was determined and used in the subsequent tests. Because of the highly sensitivity of LAMP reaction, DNA contamination and carry-over of amplified products were prevented by using sterile tools at all times, performing each step of the analysis in separate work areas,

Table 1. Sequences of LAMP primers for the amplification of the repetitive DNA sequence from *Loa loa* (GenBank Accesion No. M34259.1).

Primer[a]	Length (bp)	Sequence (5′-3′)
F3	22	TCAGCATTTATAACAAGAAGCA
B3	24	GAATTACTGTGATGGATTACTACA
FIP (F1c+F2)	40	CAGCTCCTCACTGTGGCATG-CCAAAAAACACCGATGGATG
BIP (B1c+B2)	43	TTAAGCGACTTCGTGCTGCTAC-TCAGAAAACAACACTGTATCC

[a]F3, forward outer primer; B3, reverse outer primer; FIP, forward inner primer (comprising F1c and F2 sequences); BIP, reverse inner primer (comprising B1c and B2 sequences).

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minimizing manipulation of the reaction tubes and closing them with flexible parafilm. Negative controls (ultrapure water) were included in each LAMP reaction. These controls never amplified.

Detection of LAMP product. Amplified DNA in the LAMP reaction causes turbidity due to the accumulation of magnesium pyrophosphate, a by-product of the reaction. After inactivation of the reaction tubes, the turbidity of reaction mixture was inspected by the naked eye. The LAMP amplification results were also visually detected by adding 2 μL of 1:10 diluted 10,000X concentration fluorescent dye SYBR Green I (Invitrogen) to the reaction tubes. A successful LAMP reaction would turn to green; otherwise, it would remain orange. Additionally, the LAMP products were also monitored using 2% agarose gel electrophoresis stained with ethidium bromide, visualized under UV light and then photographed using the ultraviolet (UV) image system (Gel documentation system, UVItec, UK).

Specificity and sensitivity of LAMP. To determine the specificity of the LAMP assay for L. loa, DNA samples from other parasites including *Mansonella perstans, Brugia pahangi, Strongyloides venezuelensis Anisakis simplex, Schistosoma mansoni, S. haematobium, S. intercalatum, S. bovis, Fasciola hepatica, Echinostoma caproni, Dicrocoelium dendriticum, Hymenolepis diminuta, Taenia taeniformis* and *Echinococcus granulosus* were tested as controls. The sensitivity of the assay was confirmed using genomic DNA from L. loa which was 10-fold
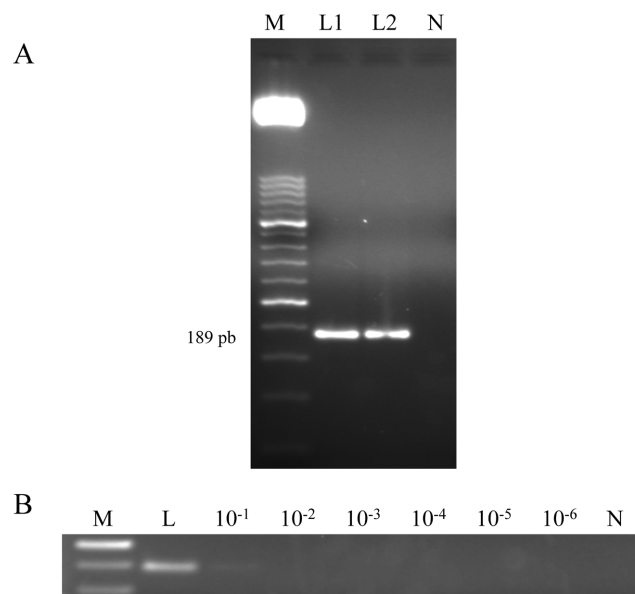

Figure 1. PCR verification and detection limit. (A) PCR verification of expected target length using LAMP outer primers F3 and B3. Lane M, 50 bp DNA ladder (Molecular weight marker XIII, Roche); lanes L1 and L2: *Loa loa* DNA; lane N, negative control (no DNA template). (B) Detection limit of PCR. Lane M, 50 bp DNA ladder (Molecular weight marker XIII, Roche); lane L: *Loa loa* DNA (5 ng); lanes 10^{-1}–10^{-6}: 10-fold serially dilutions of *L. loa* DNA; lane N, negative control (no DNA template).
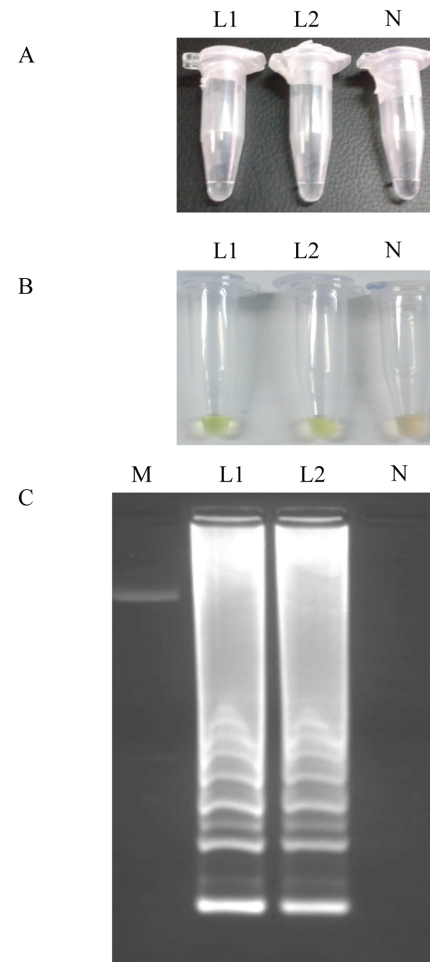
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Figure 2. LAMP detection of *Loa loa* genomic DNA samples. (A) By the naked eye; (B) by adding SYBR Green I; (C) on agarose gel stained with ethidium bromide. Lanes L1 and L2: *Loa loa* adult worm genomic DNA; lane N: no template (negative control); lane M: 50 bp DNA ladder (Molecular weight marker XIII, Roche).

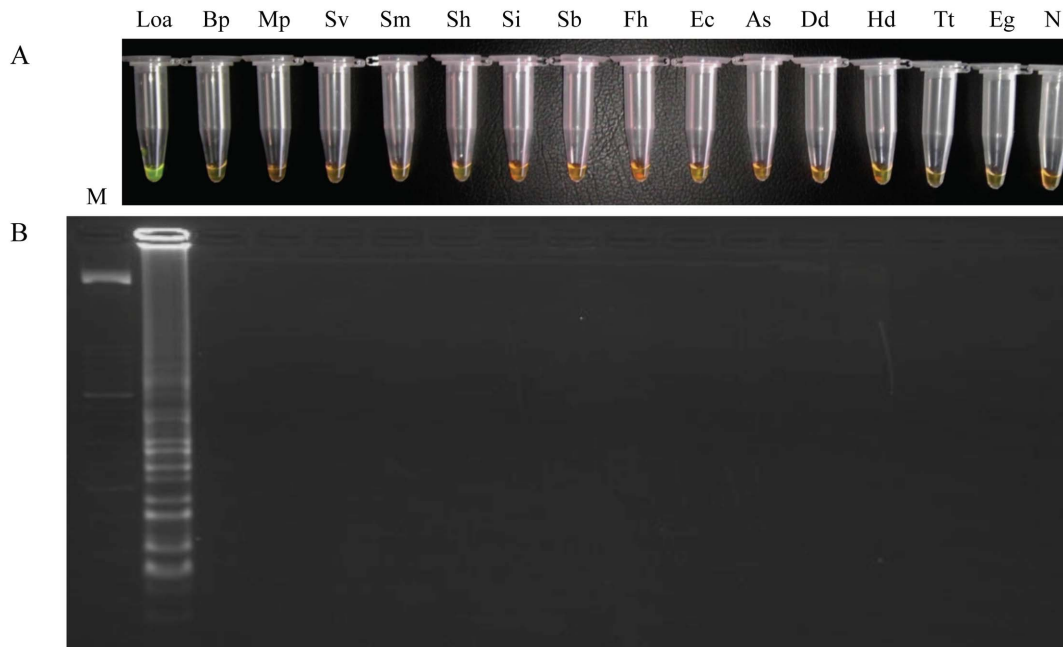doi:10.1371/journal.pone.0094664.g002

Figure 3. Specificity assessment of LAMP assay. (A) Visual examination of LAMP products by adding SYBR Green I. (B) Agarose gel electrophoresis of amplified products. Lanes Loa, Bp, Mp, Sv, Sm, Sh, Si, Sb, Fh, Ec, As, Dd, Hd, Tt, Eg: DNA from *Loa loa, Brugia pahangi, Mansonella perstans, Strongyloides venezuelensis, Schistosoma mansoni, Schistosoma haematobium, Schistosoma intercalatum, Schistosoma bovis, Fasciola hepatica, Echinostoma caproni, Anisakis simplex, Dicrocoelium dendriticum, Hymenolepis diminuta, Taenia taeniformis* and *Echinococcus granulosus*, respectively. Lane M: 50 bp DNA ladder (Molecular weight marker XIII, Roche); lane N: negative control (no DNA template).
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serially diluted as mentioned above and also with the human blood samples spiked with the same dilutions.

Results

PCR verification of target length and sensitivity

The conventional PCR amplification using outer primers F3 and B3 was used to verify whether the correct target was amplified and an expected 189 bp fragment was obtained (Fig. 1A), which is considered to correspond to the 839 bp species-specific repetitive DNA sequence from *L. loa* (GenBank Accesion No. M34259.1) (data not shown). To determine the detection limit of the PCR, a 10-fold serial dilution ranging from 10^{-1} to 10^{-6} of *L. loa* adult worm DNA was amplified. The minimum amount of DNA detectable by PCR was 0.5 ng (Fig. 1B).

Detection of LAMP product

The optimum incubation temperature for LAMP assay with the *L. loa* primer set was established using a range of temperatures (61, 63 and 65°C) for 60 min to optimize the reaction conditions and then heated at 80°C for 5 min to terminate the reaction. The LAMP assay could successfully take place at temperatures of 61°C, 63°C and 65°C. However, better results on agarose gels were obtained when using 63°C in both thermocycler and the conventional heating block (data not shown). Thus, the optimum temperature and time for LAMP were choose to be 63°C and 60 min, respectively, and used for all the following applications. Positive LAMP reactions turned turbid whereas negative ones remained clear (Fig. 2A). The original orange color of the SYBR Green I changed to green in case of positive amplification, whereas the original orange color was retained for the negative control with no amplification (Fig. 2B). After electrophoresis on

agarose gels a ladder of multiple bands of different sizes could be observed in positive samples (Fig. 2C).

Specificity of the LAMP assay

The specificity of the LAMP assay was tested using heterogeneous DNA samples as controls. Positive amplification was only observed using DNA from *L. loa*. By contrast, DNA samples from other specimens were not amplified. This result indicates that no false-positive amplifications were observed with these heterogeneous species in the LAMP assay (Fig. 3).

Sensitivity of the LAMP assay

To determine the limit of detection of the LAMP assay, a 10-fold serial dilution of *L. loa* genomic DNA was amplified by LAMP. The reaction mixtures were incubated in both thermocycler and conventional heating block to compare results. The limit of detection of LAMP using a thermocycler was 50 fg (Fig. 4A), whereas the limit of detection using a conventional heating block was established in 0.5 ag (Fig. 4B), showing that LAMP assay is much more sensitive when a heating block was used to incubate the reaction tubes.

The detection limit of LAMP in simulated human blood samples was also examined. The LAMP assay also detected the presence of DNA from *L. loa* down to as little as 0.5 ag when using a conventional heating block to incubate the reaction tubes (Fig. 5).

Discussion

In our work, a 839 bp species-specific repetitive DNA sequence from *L. loa* [10] was selected as a target for a LAMP-based method to detect purified *L. loa* DNA. To determine the specificity of *L. loa* LAMP assay, reactions were conducted for amplification of *L. loa* genomic DNA including heterogeneous DNA from other parasites

A

Loa 10⁻¹ 10⁻² 10⁻³ 10⁻⁴ 10⁻⁵ 10⁻⁶ N

M

B

Loa 10⁻¹ 10⁻² 10⁻³ 10⁻⁴ 10⁻⁵ 10⁻⁶ 10⁻⁷ 10⁻⁸ 10⁻⁹ 10⁻¹⁰ 10⁻¹¹ 10⁻¹² 10⁻¹³ N1 N2 N3
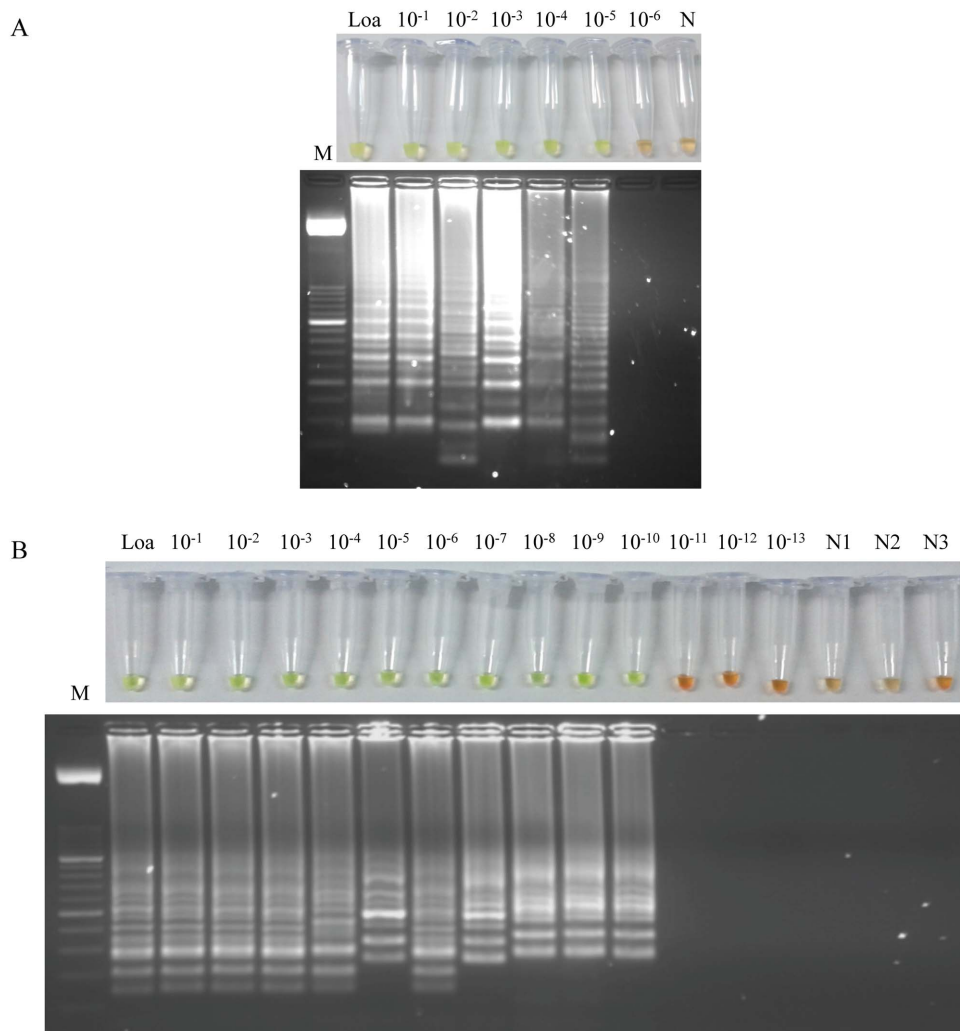
M

Figure 4. Sensitivity assessment of the LAMP assay for *Loa loa* using serial dilutions of genomic DNA by the addition of SYBR Green I or by visualization on agarose gel stained with ethidium bromide. (A) Sensitivity assessment performed with a thermocycler. (B) Sensitivity assessment performed with a heating block. Lane Loa: genomic DNA from *Loa loa* (5 ng); lanes 10^{-1}–10^{-13}: 10-fold serially dilutions; lanes N, N1, N2, N3: negative controls (no DNA template). Lane M: 50 bp DNA ladder (Molecular weight marker XIII, Roche).
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such us, *Mansonella perstans*, *Brugia pahangi*, *Strongyloides venezuelensis* *Anisakis simplex*, *Schistosoma mansoni*, *S. haematobium*, *S. intercalatum*, *S. bovis*, *Fasciola hepatica*, *Echinostoma caproni*, *Dicrocoelium dendriticum*, *Hymenolepis diminuta*, *Taenia taeniformis* and *Echinococcus granulosus*. As a result, only *L. loa* DNA was amplified, and no cross-reaction was found when using DNA as a target from other filarial parasites such as *M. perstans*, or even *B. pahangi*, which is a filarial parasite closely related to *L. loa* [22]. Moreover, a conventional PCR was performed with two outer primers to amplify *L. loa* DNA in order to confirm that the LAMP amplified the correct target, thereby ensuring high specificity for target amplification. An important advantage of LAMP is that it allows visual detection of amplification with naked eye inspection either in the form of visual turbidity [17] or visual fluorescence through the addition of fluorescent dyes such as SYBR Green I [23]. In our experiments using LAMP to amplify *L. loa* DNA not only a change in turbidity was clearly visually appreciated but also a visual detection of an orange-to-green color change with SYBR Green I was easily accomplished and always correlated with gel electrophoresis findings.

Regarding the sensitivity of LAMP, it is generally considered that LAMP is highly sensitive compared to PCR methods. In our work, the sensitivity of both conventional PCR using outer primers and LAMP assay was evaluated using *L. loa* genomic DNA which was serially 10-fold diluted. The detection limit of PCR was 0.5 ng of *L. loa* DNA whereas the detection limit of LAMP assay using a thermocycler was 50 fg, showing that LAMP assay is more sensitive than PCR method. Unexpectedly, when LAMP assay was performed in a conventional heating block, the results showed that 0.5 ag of *L. loa* DNA could be detected. Additionally, we obtained identical results when simulated human blood samples spiked with *L. loa* genomic DNA 10-fold serially diluted were used to set up the sensitivity of LAMP. Thus, LAMP assay using thermocycler was 10^4 times more sensitive than a conventional PCR whereas it was 10^9 times more sensitive when a conventional heating block was used for amplification. Increasing in sensitivity of LAMP assay when a heating block instead a thermocycler is used to keep both temperature and time for amplification has been observed by us in developing of other LAMP assays in which we are currently working (unpublished data).
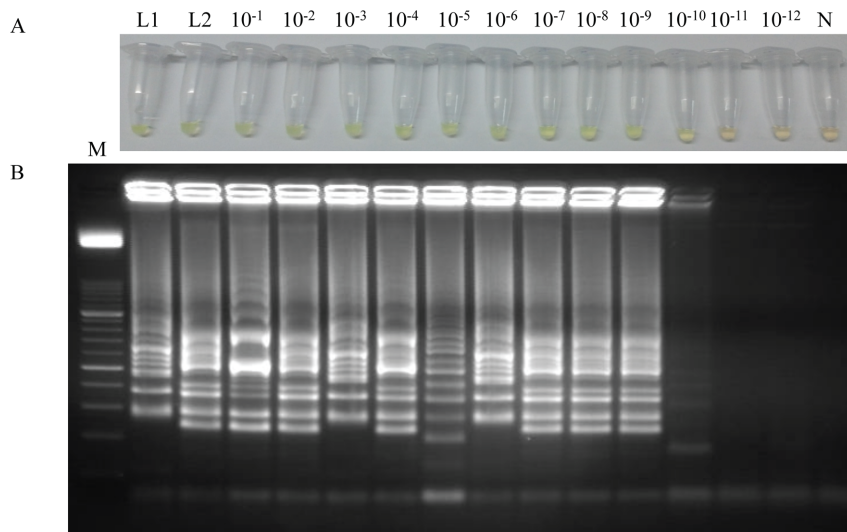
Figure 5. Sensitivity assessment of the LAMP assay for *Loa loa* performed with a heating block using simulated human blood samples spiked with serial dilutions of genomic DNA by the addition of SYBR Green I (A) or by visualization on agarose gel stained with ethidium bromide (B). Lane L1: genomic DNA from *Loa loa* (5 ng) as positive control; lane L2: human blood sample spiked with 5 ng of genomic DNA from *Loa loa*; lanes 10^{-1}–10^{-12}: human blood samples spiked with 10-fold serially dilutions; lane N: negative control (no DNA template). Lane M: 50 bp DNA ladder (Molecular weight marker XIII, Roche).
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It seems logical to consider that using a thermocycler or a heating block to keep both temperature and time for LAMP amplification should not influence on different sensitivities obtained by the technique; however, to use a simple and cheaper incubator could be better to obtain a higher sensitivity. It is known that *Bst* polymerase Large Fragment used in typically LAMP assays for DNA amplification has a 100% activity at 60°C–65°C, but it also has 20% activity up to 70°C. When a quality thermocycler is used for LAMP both temperature and time for DNA amplification can be accurately pre-programmed (*i.e.* 60 min at 63°C; 5 min at 80°C) and the cycler raises and lowers temperature of the block to achieve fast temperature changes in a scheduled time. However, when an economical heating block is used for LAMP, time programming and fast temperature changes are not possible. Once the reaction tubes have been incubated for 60 min at 63°C, a manually change to increase temperature to achieve heat-inactivation of the *Bst* polymerase (greater than 70°C; typically 80°C for 5–10 min) is required. That temperature increase is commonly slow as the block is heating up gradually and may take some time to get heat-inactivation of the *Bst polymerase*, thus enabling the enzyme to work for longer at a range of temperatures from started 63°C up to 70°C; in this timeframe the *Bst polymerase* has certain activity before it becomes heat-inactivated and, therefore, still yielding amplification.

The detection limit obtained with our primer set designed for LAMP to detect *L. loa* genomic DNA (0.5 ag) was found to be higher than one obtained by real-time PCR (0.1 pg) described by Fink *et al* (15) and also than those obtained by other LAMP assays designed for DNA detection from other pathogenic filarial worms. Thus, a LAMP diagnostic assay for *brugian filariasis* detected 1 pg of total genomic DNA purified from *Brugia malyi* worms (9 times less sensitive) [20]; other LAMP assays designed to detect DNA from *Wuchereria bancrofti* [21] and *Dirofilaria immitis* [19] have reported a detection limit of 0.1 pg of filarial DNA (6 times less sensitive). It has been estimated that a single microfilaria contains approximately an amount of 100 pg of DNA [24,25,26]. Using our LAMP assay it is possible to detect as little as 0.5 ag of total genomic DNA

purified from *L. loa* worm which is equivalent to 1/1.000.000[th] of a microfilaria.

We are aware that our LAMP assay has not been tested with clinical samples, but our data obtained in simulated human blood samples indicate that the LAMP assay designed is sensitive enough to detect *L. loa* at a very low level, and thus the use of this technique may have important clinical applications. It has been reported that the sensitivity of LAMP was less affected by the various components of the clinical samples than was PCR and, moreover, DNA purification from samples could be omitted [27]. In this context, and considering the high sensitivity obtained in detecting *L. loa* DNA in simulated human blood samples, our assay could be potentially use in screening clinical samples either with extracted DNA or just with boiled whole blood.

In conclusion, the results of the current study demonstrated that the established LAMP assay is cost-effective, specific and very highly sensitive technique for detection of *L. loa* DNA. Although further research for evaluation of the method for the application in clinical samples is required, the method is potentially adaptable for field diagnosis and disease surveillance in loiasis-endemic areas.

Acknowledgments

Author Contributions

Conceived and designed the experiments: PFS AM MCS JLA JPA. Performed the experiments: PFS POM BVS. Analyzed the data: PFS POM AM JLA JPA MCS. Wrote the paper: PFS.

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