The development of NESTED-LAMP method as a novel approach for diagnostic purposes while using Leishmania parasite as the study model

Ava Yazdanpanah
Fasa University of Medical Science
Sahar Samsami
Fasa University of Medical Science
Abdolmajid Ghasemian (majidghasemian86@gmail.com)
Karaj Islamic Azad University Faculty of Sciences https://orcid.org/0000-0002-1243-6341
Sahar Namavari
Fasa University of Medical Science
Saeed Ataei
Fasa University of Medical Science
Neda Sepahi
Fasa University of Medical Science
Negin Namavari
Fasa University of Medical Science
Ali Ghanbariasad
Fasa University of Medical Science

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Abstract

Loop-mediated isothermal amplification (LAMP) does not require thermal cycler machines to amplify DNA and has the potential to revolutionize molecular biology by bypassing the need for sophisticated equipment. The method also has a low running cost and short turnaround times with higher sensitivity when compared with many other amplification methods. The objective behind this study was to introduce a new platform for LAMP assay with improved sensitivity. In order to do such, *Leishmania* spp parasites were used as the study model for the investigation purposes. A new primer set (four primers plus two extra ones) were designed and optimized for the novel LAMP assay which we further assigned the name “NESTED-LAMP”. The primers target included ITS1 gene locus of cutaneous leishmaniasis (CL) to enhance the assay sensitivity. The minimum of detection of this new assay was further compared with the traditional LAMP assay, polymerase chain reaction (PCR), and real-time PCR (RT-PCR) methods. NESTED-LAMP detected 1 parasite/μL while the conventional counterpart technique was able to detect 10^5 parasites/μL. The sensitivity of PCR and RT-PCR declined when the concentration of parasite/μL was below 10^5 and 10^2, respectively. Furthermore, this study demonstrated 100% specificity and sensitivity regarding the NESTED-LAMP method. This method could be employed as a suitable alternative for diagnostic purposes as the results demonstrated a 10^5-fold improvement in sensitivity for the NESTED-LAMP approach compared with the conventional counterpart technique. Also, the cost-efficiency with higher sensitivity and specificity ensures the accuracy of the results.

Introduction

Accurate diagnostic tests have a key role in patient management, treatment and the control of most infectious diseases (Peeling, Smith et al. 2010). Molecular approaches have now become widely established for the detection of causal agents of infections which arise from various sources (viral, bacterial, fungal, and protozoa). Polymerase chain reaction (PCR) has become the method of choice for amplifying nucleic acids since it was developed by Carry Mullis et al (Mullis, Faloona et al. 1986, Mullis 1990). PCR-based diagnostics have been effectively developed for a wide range of microorganisms. Due to its high sensitivity, specificity, and speed of amplification, PCR has been preferred to identify non-cultivable organisms *in vitro*, or for instances when existing cultivation techniques are not sensitive enough or entail prolonged incubation periods rendering cultivation an unsuitable approach (Louie, Louie et al. 2000, Post and Ehrlich 2000). Despite its popularity for diagnostic application, PCR amplification harbors several inherent drawbacks such as the production of non-specific products due to high DNA similarities amongst species which inadvertently leads to an insufficient amplification of target genes for diagnostic purposes. A PCR test also demands expensive thermal cyclers, dedicated space, trained personnel and time-consuming post-PCR analysis, which are not desirable. The emergence of several innovative variants of PCR such as nested PCR (nPCR) and real-time PCR (RT-qPCR) are further aimed to resolve the shortcomings associated with the conventional PCR (Foo, Nurul Najian et al. 2020). RT-qPCR has numerous advantages over the conventional PCR methods in terms of time efficiency, quantitative measurement, lower contamination rate, higher sensitivity and specificity, and easy standardization

(Jozefczuk and Adjaye 2011, Navarro, Serrano-Heras et al. 2015). However, the main RT-qPCR drawback includes machine-dependency being expensive which requires regular maintenance (Jiang, Liang et al. 2016).

Within the past decade, numerous isothermal amplification techniques have been established. These include recombinase polymerase amplification, rolling circle amplification and helicase-dependent amplification (Zanoli and Spoto 2013). Notomi et al. (Notomi, Okayama et al. 2000) successfully devised a novel isothermal amplification method with which a low copy-number DNA was amplified into million copies within an hour. The method was introduced as loop-mediated isothermal amplification (LAMP), a technique which bears several advantages. First and foremost, the reaction takes place at isothermal temperatures between 60°C and 65°C, therefore simple incubators, such as a water bath or block heaters, are suitable for the amplification process to take place. Secondly, the process does not require any DNA template preparation and has the potential to generate 10^9 copies of DNA, which is highly beneficial in terms of efficacy (Dhama, Karthik et al. 2014). Hence, LAMP has been advocated as a low-cost genetic analysis tool for resource-poor settings (Neonakis, Spandidos et al. 2011, Abdul-Ghani, Al-Mekhlafi et al. 2012). The high specificity of this test is due to the employment of two pairs of primers which are designed to specifically recognize six distinct regions of the target DNA (Notomi, Mori et al. 2015). In addition, the sensitivity along with amplification efficiency of LAMP are substantially higher than that of PCR (or RT-qPCR) (Jang, Lim et al. 2021). Although the sensitivity of LAMP might not surpass that of RT-qPCR, it is more specific (Abdul-Ghani, Al-Mekhlafi et al. 2012). Various genetic markers, which differ in copy-number and nucleotide sequence, have been used in various studies in order to identify different species of *Leishmania*. The usage of genes with high copy-number rather than those of single-copy are more preferred as it increases the sensitivity (Akhoundi, Downing et al. 2017). The precision of diagnostic assays is dependent on the two criteria namely sensitivity (i.e., true positive rate) and specificity (i.e., true negative rate) (Fleuren, Klausch et al. 2020), hence we envisioned an improved version of the conventional LAMP assay which demonstrated enhanced sensitivity and was further entitled NESTED-LAMP.

The sensitivity and specificity of NESTED-LAMP were investigated using an ITS1 marker from *Leishmania major (L. major)* as the causative agent of cutaneous leishmaniasis (CL), which accounts for the highest share of the disease worldwide (Mahmoudzadeh-Niknam, Ajdary et al. 2012, Koltas, Eroglu et al. 2014, Mokni 2019, Abbas, Lachheb et al. 2022). In this study, we opted for the ITS1 marker due to its high copy-number (≈ 200) and conservation in *L. major*. The results were finally compared with common molecular diagnostic methods.

Materials and Methods

Collecting patient samples

A total of 76 samples were taken from the margin of the leech wounds, which were reported positive using microscopic analysis, and 16 samples from the margin of wounds caused by agents other than the

Leishmania were chosen as negative samples.

Collecting positive and negative control samples

Standard samples of *L. major* parasite (MRHO / IR / 75 / ER) and *L. tropica* (MHOM / SU / 74 / K27) were obtained from the Pasteur Institute of Iran. The standard samples of *L. infantum* species (MCAN / IR / 97 / LON490) with DNA of *Toxoplasma gondii*, *Cryptosporidium parvum*, *Candida albicans* and *E. coli* were acquired from the Shiraz University of Medical Sciences.

DNA extraction from clinical and standard samples

Samples were subjected to DNA extraction according to the FlexiGene DNA Kit protocol (Qiagen, Cat. No. 51204, Hilden, Germany). The extracted DNA was analyzed using a nanodrop spectrophotometer (Biotech, Synergy HTX).

Standard strain cultivation

The standard strain of *L. major* with identification code (MHOM / IR / 75 / ER), was cultivated using RPMI 1640 medium (Thermo Fisher Scientific, US), supplemented with 15% FBS (Thermo Fisher Scientific, US), 100 IU/mL of penicillin and 100 µg/mL of streptomycin (Gibco, Thermo Fisher Scientific, US) and were stored at 25°C in a shaker incubator (TiaTech-BR-12SH).

Primer design: To design LAMP and Nested-LAMP primers, the ITS1 conserved sequences of *L. major* (accession number: NC_007268.2) were first extracted from National Center for Biotechnology Information (NCBI) data bank (https://www.ncbi.nlm.nih.gov/). Then, LAMP and Nested-LAMP primers were designed using Primer Explorer V5 online software (http://primerexplorer.jp/e/). 5'-TGA TAC CAC TTA TCG CACT T-3' sequence (Monroy-Ostria, Nasereddin et al. 2014) was selected as primer BA (B additional). Finally, primers' specificity was confirmed via MEGA5, BLAST and Gene Runner software (Fig. 1). Table 1 illustrates the primers used in this study for PCR and RT-qPCR analysis (de Almeida, Koru et al. 2017). F3 LAMP and B3 LAMP were used as the external primers. FIP LAMP was the forward internal primer made of F1 LAMP and F2 LAMP regions. BIP LAMP served as the backward internal primer being composed of B1 LAMP and B2 LAMP regions. F cloning and B cloning were the primers used to clone the target sequence into the pTZ57R / T vector. F real-time and B real-time were the primers used for RT-qPCR analysis. F PCR and B PCR were the forward and backward primers used to amplify the ITS1 sequence, respectively.

NESTED-LAMP optimization and analysis

The Nested-LAMP reaction (Table 2) was designed by modifying the conventional LAMP assay (Table 3). An additional pair of primers were added to the primers used in the conventional LAMP. The reaction mixture was supplemented with the internal and external (which is used in conventional LAMP method) primers 20 minutes after the process had begun. The experiment was carried out at different

temperatures and durations to determine the optimum conditions. The time-point when it was best to add the primers was investigated.

Table 2
Nested-LAMP setup condition.

Materials	Amount
Betaine 5mM	4µl
dNTP 40mM	0.75µl
Buffer 10X	2.5µl
Bst DNA Polymerase 8000U/ml	0.8µl
Mgso4 100mM	1.5µl
F3,B3(10µM)	0.3µl
F_A,B_A (10µM)	0.6µl
FIP,BIP (10µM)	3.2µl
DNA	1µl
DW	10.35µl
Total volume	25µl

Table 3
Conventional LAMP setup condition.

Materials	Amount
Betaine 5mM	4µl
dNTP 40mM	0.75µl
Buffer 10X	2.5µl
Bst DNA Polymerase 8000U/ml	0.8µl
Mgso4 100mM	1.5µl
F3,B3(10µM)	0.3µl
FIP,BIP (10µM)	3.2µl
DNA	1µl
DW	10.95µl
Total volume	25µl

Reaction mixture with a final volume of 25 µL included: betaine (4 µL), 2.5 µL of ThermoPol Reaction Buffer 10X (New England Biolabs, Ipswich, MA; containing Tris–HCl (20 mM), (NH4)2SO4 (10 mM), KCl (10mM), MgSO4 (2 mM), Triton X-100 (0.1%), pH 8.8, 25°C, dNTP (0.75 µL), $MgSO_4$ (1.5 µL), Bst polymerase large Fragment (0.8 µL) (New England Biolabs, Ipswich, MA), primer F_3/B_3 (0.3 µL), primer FIP/BIP (3.2 µL) and DNA (1 µL).

In order to increase the sensitivity, in addition to the conventional LAMP primers, two primers (FA and BA) with a final concentration of 0.6 µL of 10 µM solution were used. The remaining primers were added to 20 minutes after the reaction initiation. CYBER green I at 0.5µL (1:10) was added to the reaction product. The green-yellow color represented a positive result and the orange color represents a negative result.

Gene cloning

A 936bp fragment was amplified using the primers of table 1. After electrophoresis and DNA extraction via Expin Combo GP, mini Kit (GeneAll, South Korea), the product was cloned into pTZ57R / T vector using the InsTAclone PCR Cloning Kit (Thermo Fisher Scientific, Cat. No. K1214). Blue and white selection was performed when the *E. coli* (DH5α) was transformed with the construct. Once the plasmids were once again extracted, cloning was confirmed via sequencing (Microsynth Switzerland).

Determining the sensitivity of NESTED-LAMP, PCR, Real-Time PCR and LAMP methods: The vector (1000ng) carrying the target sequence was serially diluted up to 10-fold. Each dilution was further subjected to NESTED-LAMP amplification which was carried out in a water bath at 60°C for 2h. After the amplification had finished, each reaction was supplemented with 0.5 µL of 10-fold diluted SYBER green I (Thermo-Fisher Scientific, Grand Island, NY). In addition, each dilution was subjected to conventional LAMP, PCR (Table 4) and Real-time PCR (Table 5) amplifications.

Table 4
PCR setup condition.

Materials		Amount		
DNA	1 µl	Primary denaturation	C°95	5 min
Master mix 2x	10 µl	denaturation	C°94	30 s
Primer	2 µl	Annealing	C°45	30 s
DW	7 µl	Primary Extension	C°72	45 s
Total volume	20 µl	Final Extension	C°72	5 min
		Cycle	30	

Table 5
Real- time PCR setup condition

Materials		Amount		
DNA	1 µl	Primary denaturation	C°95	5 min
Master mix 2x	7.5 µl	denaturation	C°94	15 s
Primer	1 µl	Annealing	C°50	15 s
DW	5.5 µl	Primary Extension	C°72	15 s
Total volume	15 µl	Final Extension	C°72	5 min
		Cycle	30	

The test was carried out according to the number of materials listed in Tables 4 and 5 for 120 minutes at 60 ° C using a water bath.

PCR was performed according to the number of materials listed in Tables 4 and 5 in order to determine the sensitivity of the technique using serial dilution.

The test was performed to determine the sensitivity of the technique using serial dilution.

Determining the specificity of PCR, Real-Time PCR, LAMP and NESTED-LAMP methods

In order to confirm the specificity of the Nested-LAMP technique, tests were performed on negative control samples which were*Leishmania infantum, Leishmania tropica, Toxoplasma*, *Cryptosporidium* and *E. coli*, the conditions of each sample can be described in Table 2. The results were analyzed by adding CYBER green I at the end of each test. Also, the standard sample of *Leishmania major* parasite was used as a positive control sample to detect false-negative results.

Clinical sample analysis

After DNA extraction from samples of patients suspected of CL disease, the Nested-LAMP technique was employed to investigate 76 samples according to the previous conditions. The results were further analyzed when test tubes were supplemented with CYBER green I. For comparison, PCR, Real-Time PCR, LAMP methods were performed on all clinical samples.

Results

The minimum of detection (MOD) of the NESTED-LAMP method

Each dilution was subjected to PCR and Real-Time PCR analysis. The results of NESTED-LAMP (Fig. 2) and conventional LAMP (Fig. 3) were analyzed using a fluorescent dye (CYBER green I). The green color indicated a positive result and orange color was indicative of no DNA amplification.

The results of NESTED-LAMP and conventional LAMP showed that the NESTED-LAMP method could detect 1fg/µL of plasmid which was approximately equivalent to 1 parasite/µL (Fig. 4). On the other hand, the conventional LAMP method was able to detect plasmid concentrations above 1–10 ng/µL, which was roughly equivalent to 10^5 parasites/µL (Fig. 5).

The MOD of PCR and Real-Time PCR were revealed to be 1 ng/µL and 1–10 pg/µL of plasmid, which were equivalent to roughly 10^5 parasites/µL, and 10^2 parasites/µL, respectively (Fig. 4).

Sensitivity and specificity of PCR, Real-Time PCR, LAMP and NESTED-LAMP methods

The NESTED-LAMP demonstrated high specificity and sensitivity as no false-positive or false-negative result was detected. The blood samples which were previously reported positive via microscopic evaluation were determined to be positive (green color) using the NESTED-LAMP method. Along with that, the negative control samples remained negative (orange color) via NESTED-LAMP. Therefore, Sensitivity and Specificity were approximately 100% (Table 6).

Table 6
A comparative analysis of clinical samples via various approaches.

PCR		Real-Time PCR		LAMP		NESTED-LAMP	
Clinical Samples (16 negatives controls and 76 positives)							
NC	P	NC	P	NC	P	NC	P
ND	64	ND	70	ND	66	ND	76
(Sensitivity 84% and specificity100%)		(Sensitivity 92% and specificity 100%)		(Sensitivity 86% and s specificity 100%)		(Sensitivity 100% and specificity 100%)	

There were 16 negative control samples along with 76 microscopically evaluated positive samples were subjected to analysis. All methods showed 100% specificity; however, the highest sensitivity was produced via the NESTED-LAMP method. NC: negative control P: positive and ND: not detected.

Discussion

There are several diagnostic approaches on the market to detect *L. major* (Chaouch, Mhadhbi et al. 2013, de Vries, Reedijk et al. 2015); however, there are some shortcomings (Anversa, Tiburcio et al. 2018) to which our goal was to reduce or alleviate. Although the traditional diagnostic methods namely PCR and RT-qPCR are highly popular and convenient, they are still costly as both demand sophisticated laboratory facilities (Torres-Guerrero, Quintanilla-Cedillo et al. 2017). The optimization of these tests are an arduous task and becomes time-consuming. LAMP assays are becoming increasingly popular as they are

straightforward to perform, cost-efficient, and easily optimized (Notomi, Mori et al. 2015, Wong, Othman et al. 2018). However, there have been some downsides regarding the sensitivity of the tests (Verma, Singh et al. 2017, Adams, Schoone et al. 2018, Waliullah, Ling et al. 2020). In order to address the issues, we envisioned that through an additional primer pair and optimizing the inoculation time of the primers, sensitivity could be improved. We speculated that if the target template would exist in high quantities, the detection range would improve, covering the samples with extremely low quantities of the target sequence. The in the quantity results in an increase in precision, resulting in an increase in power and decrease in false negatives. A decrease in false negatives, thus results in an increase in sensitivity. Thereby, we introduced a new pair of primers (FA and BA) which are engulfed in the sequence where the primers of the conventional LAMP bind. By amplifying a larger fragment which encompasses the target sequence for 20 minutes before the addition of LAMP primers, determined throughout the optimization tests, samples containing low quantities of the target sequence, which used to be negative using conventional LAMP, were found positive. In this study, we demonstrated that the NESTED-LAMP approach has approximately a 10^5-fold higher sensitivity when compared to the conventional LAMP as it could detect the minute concentration of *Leishmania* parasite at 1fg/µL. Further analysis revealed that the NESTED-LAMP method surpassed the gold standard approaches namely PCR and Real-time PCR in terms of sensitivity by a staggering 10^5 and 10^3 fold, respectively. Although there have been attempts to detect *L. major* using conventional LAMP assay with a low threshold of detection, the NESTED-LAMP method demonstrated better performance. For instance, in one study the conventional LAMP method was employed to detect *L. major* using cysteine protease B (cpb) as the target gene. The threshold of detection was determined to be 20 fg/µL which was 20 fold higher than that of the NESTED-LAMP method (Chaouch, Aoun et al. 2019). In another study, *L. major* was detected via conventional LAMP using KDNA as the target sequence. Despite the high copy number of KDNA, the threshold of detection was significantly higher than that of NESTED-LAMP, as the former's minimum of detection was 100 pg and that of the latter was 1 fg (28).

Conclusions

NESTED-LAMP approach demonstrated to be a suitable alternative to traditional approaches for the CL diagnosis.

Declarations

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Availability of data and materials

Not applicable

Authors' contributions

Ava Yazdanpanah: Methodology, Validation, Formal analysis

Sahar Samsami: Methodology

Saeed Ataei: Writing the original draft, designing tables, Investigation

Neda Sepahi: Methodology, Validation, Formal analysis

Negin Namavari: Writing the method section, Methodology

Ali Ghanbariasad: Conceptualization, Methodology, Supervision, corresponding author

Abdolmajid Ghasemian: Draft edition

Ethics approval and consent to participate

The study was approved by the review committee of Fasa University of medical science

Declaration of Competing Interests

The authors have no conflicts of interest to declare

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Figures

Figure 1

Primer design. A graphic illustration of primers and their respective localization

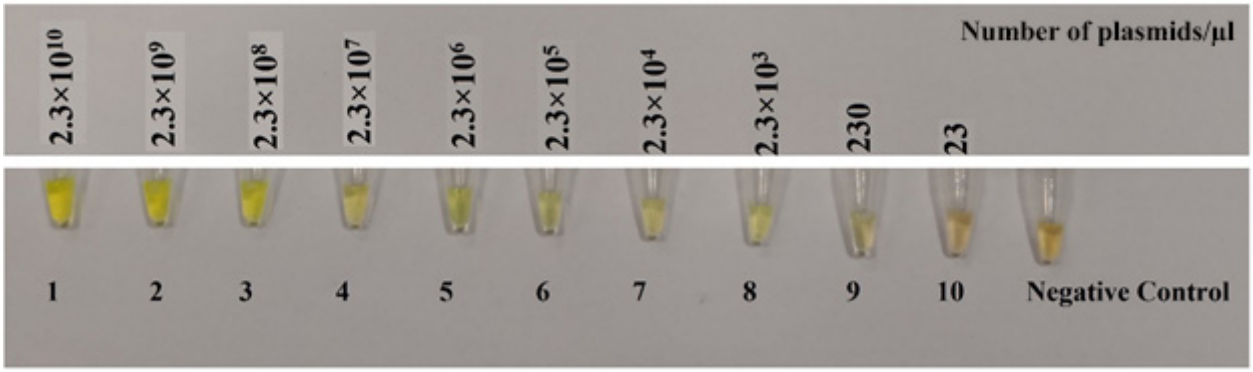

Figure 2

Detection of target sequence via NESTED-LAMP. The test was positive up to dilution number 9 (230copy per µL); however, no detection was observed in dilution number 10 (23copy per µL). The negative control sample did not reflect any color change.

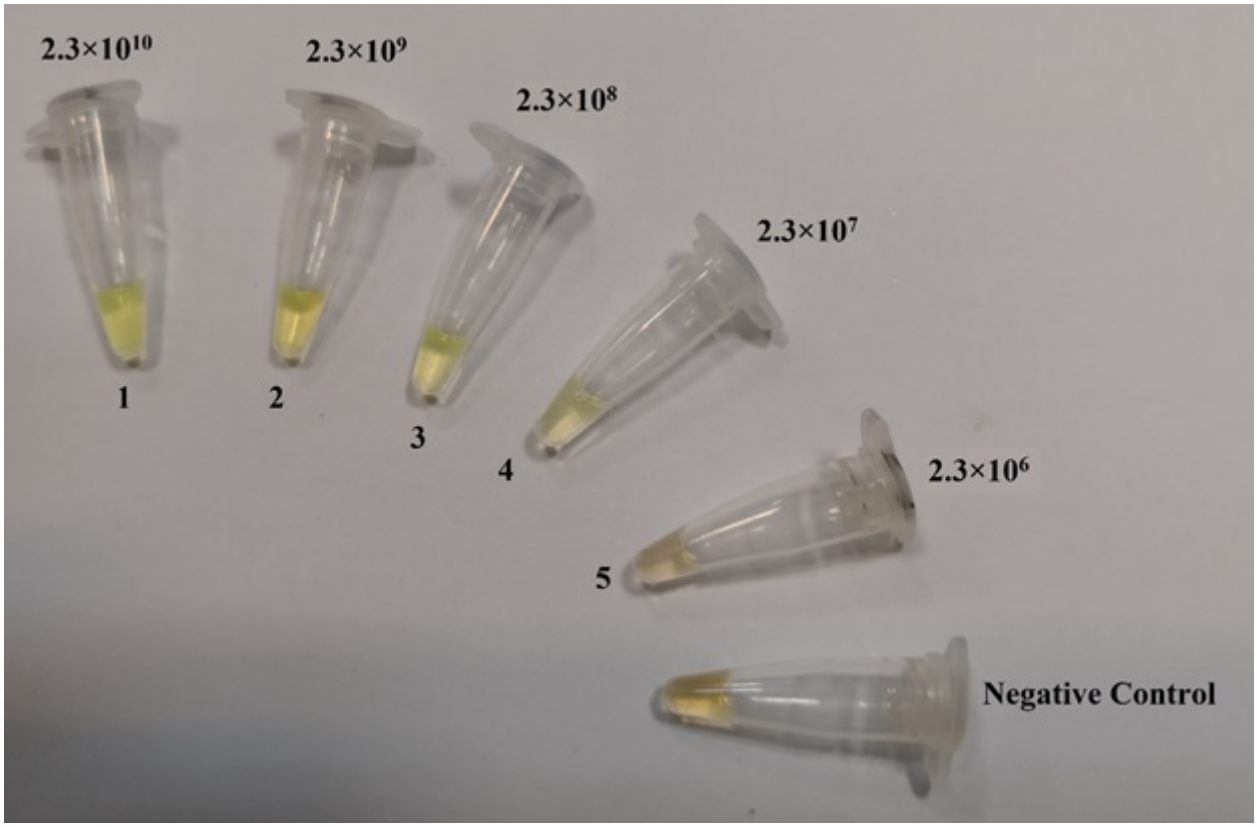

Figure 3

Detection of target sequence via conventional LAMP. The test was positive up to dilution number 4(2.3×10^{7}copy per µL); however, no color change was observed in dilution number 5(2.3×10^{6}copy per µL). The negative control sample remained negative.

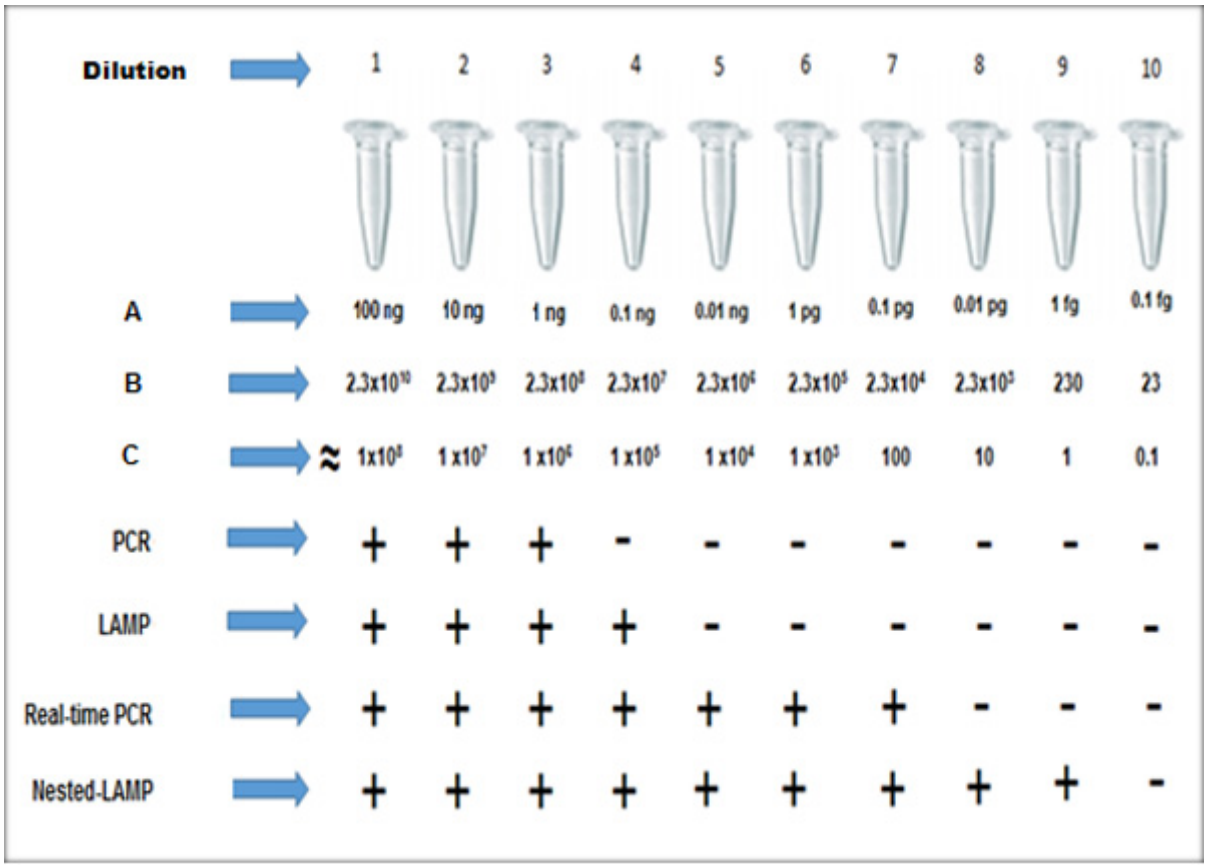

Figure 4

Determining the sensitivity of molecular tests performed based on the number of parasites using the calculation of the number of plasmids in each dilution. PCR showed the lowest detection cutoff while NESTED-LAMP was the highest A. Plasmid concentration per microliter B. Number of plasmids per microliter C. Equivalent parasite number.