Loop-mediated isothermal amplification for protozoan diagnosis: Review of ongoing development and implementations

Review Article

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ABSTRACT

Parasitic protozoa are emerging as global health risks worldwide. Accurate screening of protozoal infection is pivotal for an effective strategic control process. Traditional parasitological techniques, immunodiagnostics and PCR-based methods are the classical tools for diagnosing protozoal infection, even so they are labor and time-intensive, expensive, and occasionally give inconsistent results. The loop mediated isothermal amplification (LAMP) assay, is a single-step amplification reaction that is an extremely sensitive, efficient and robust technique for identification of protozoa. This review aims to offer an overview of the present status of LAMP technology in protozoan diagnosis and to highlight this innovative tool and its future progress through comparison with other traditional diagnostic techniques. Moreover, LAMP essential principles were described and its fundamental goals, *i.e.*, sensitivity, specificity, and overall diagnostic accuracy in comparison with conventional diagnostic techniques for protozoan infections were discussed.

Keywords: amplification; DNA; isothermal conditions; LAMP; molecular diagnostics; PCR; primers; protozoa.

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INTRODUCTION

Protozoa are among a diverse group of microorganisms that can lead to systemic infection in immunosuppressed patients. Among the protozoa that are frequently responsible for disease in immunodeficient hosts is *T. gondii*[1]. Although there are advances in the era of diagnosis, there is not a reliable and perfect point-of-care diagnostic technique. Parasite morphological identification is commonly employed in clinical diagnosis, but it has many drawbacks as it shows low sensitivity, specificity and requires significant quality and expertise. Serological diagnosis is an alternative diagnostic approach applied to avoid missed diagnosis, particularly in low-intensity of infection. Among assays used in serological diagnosis, ELISA is the most applied in diagnosis of parasitic diseases. However, its challenging drawbacks are the differentiation between past and current infections, the relatively high rate of false-positive results, and its cross reactivity. Molecular profiling, by a range of PCR based methods, DNA, and protein microarrays have started a novel era for detection of protozoan nucleic acids. Therefore, LAMP has emerged as a robust substitute technique of PCR for protozoa identification with high sensitivity and specificity[2].

Principle of LAMP

This new method for amplifying nucleic acids, utilizes Bst DNA polymerase with strand displacement activity and a range of four to six primers that are specific to 6-8 target sites. There are two inner and two outer primer sets, the inner primers include the forward inner primer (FIP) and the backward inner primer (BIP); and the outer primers are termed outer forward (F3) and outer backward (B3)[3]. Two additional forward and backward loop primers (LF and LB) can be added to accelerate the process[4]. The initial step is performed at 65°C, during which the forward inner primer hybridizes to the target DNA forming the complementary strand. The F3 primer starts strand displacement of the elongated FIP primer, resulting in release of a FIP-linked complementary strand, forming a template for BIP primer. The BIP primer starts strand synthesis at the single stranded DNA which is later displaced by B3 primer forming dumbbell-like DNA fragments which is the starting point for exponential amplification (Fig. 1). The self-priming generates long amplicons with cauliflower-shaped structures that have several loops. The advantages of LAMP are numerous and excellent. It can be conducted without the need for expensive thermocyclers, as required for PCR, because LAMP needs only a heated water bath or thermal block. It can be conducted at an isothermal temperature of 60–65°C with a short reaction time of up to half an hour. It is highly sensitive because it can detect up to one femtogram (fg) of DNA. LAMP amplicons can be identified using several methods including turbidity (visual), real-time, dyes, metal indicators and electrophoresis[5].

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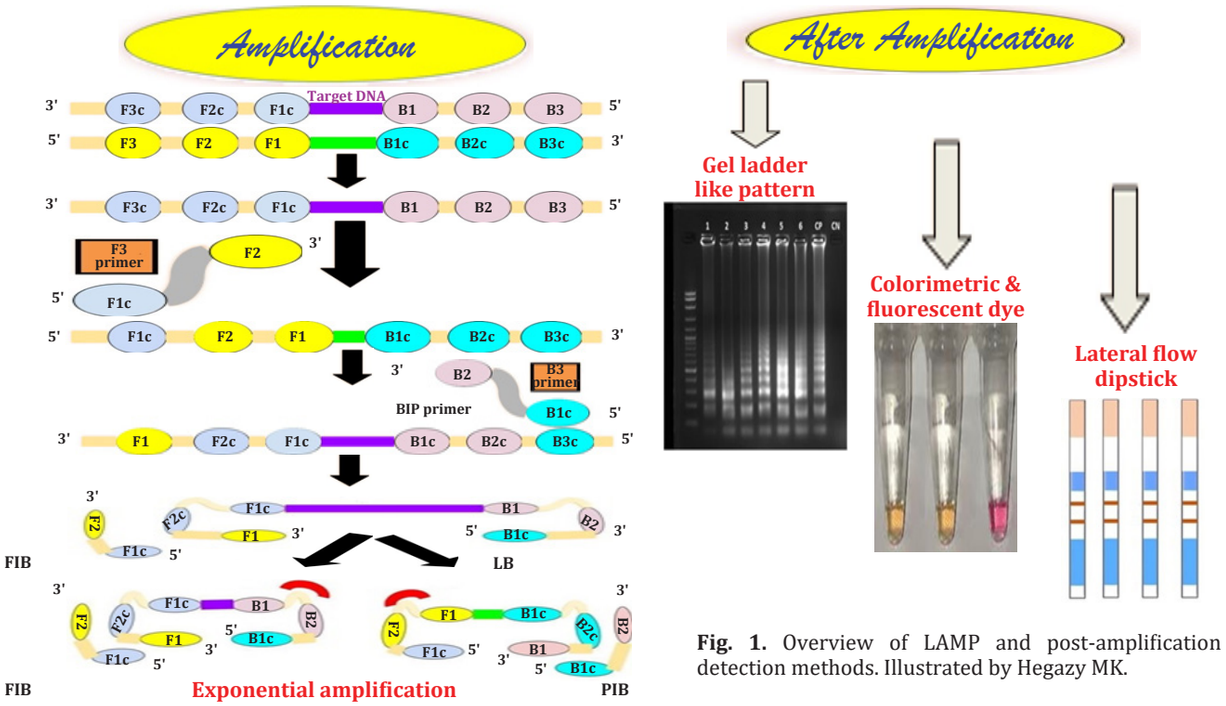

Fig. 1. Overview of LAMP and post-amplification detection methods. Illustrated by Hegazy MK.

This comprehensive review seeks to validate the potency of LAMP as a molecular identification procedure in diagnosing protozoan infections. Inclusion criteria for the review were studies dedicated to LAMP assays for protozoan identification in human, animal, or environmental specimens. Exclusion criteria included studies without original data, or those using LAMP outside the scope of protozoan detection. The databases searched included PubMed and Web of Science, covering the last ten years. The review included various studies, comprising different regions and study designs.

Plasmodium spp.

Malaria is among the most significant parasitic infections affecting humans. Among the four *Plasmodium* spp. that cause disease in humans, *P. falciparum* is responsible for the majority of severe and fatal cases[6]. The emergence of artemisinin resistance in *P. falciparum* poses a growing international risk. In Southeast Asia, the *kelch13* (*k13*) gene C580Y mutation was identified as the primary marker responsible for artemisinin resistance. Accordingly, a rapid and effective method for detecting this mutation was crucial for ongoing field surveillance. The study developed a novel diagnostic approach for detecting the C580Y mutation by LAMP coupled with integration of the MinION nanopore sequencer. The LAMP assay demonstrated a detection sensitivity of ten copies of plasmid controls, and the process was completed within 60 min. The LAMP amplicons were then sequenced to analyze the gene's nucleotide sequences within 30 min. Clinical evaluation of the LAMP assay on thirty-four *P. falciparum* blood samples from Indonesia showed 100% detection sensitivity. Sequencing of LAMP products from twelve samples by MinION, produced agreeable results with those obtained through conventional PCR and Sanger sequencing[7].

Results of another research highlighted the effectiveness of *in situ* LAMP in diagnosing *Plasmodium* infection. Suspensions of red blood cells (RBCs), consisting of RBCs infected with the *P. falciparum* 3D7 line were distributed onto plates made of cyclic olefin copolymermade hydrophilic through ion-assisted reactive etching. The suspensions were left undisturbed for 10 min to permit the RBCs to deposit on the plate surface. After washing the plate with RPMI 1640 culture medium, RBCs monolayers covered nearly the whole surface of the plate, and a hair dryer was used for drying. Formalin was used for fixation and Triton X-100 for permeabilization. Subsequently, 18S rRNA gene of *P. falciparum* was amplified, incorporating digoxigenin-tagged dUTP and a designed primer set. Fluorescence-positive infected RBCs were visualized by fluorescent microscopy[8].

Assessment of LAMP technique was applied to investigate 38 EDTA blood specimens from 23 patients, all of whom had been screened for disease using microscopic examination, antigen-RDT, and qPCR. The specimens consisted of blood with low-density infection and cases with conflicted results of diverse techniques. The LAMP method identified infection in 27 out of 28 specimens that were tested positive by qPCR. Among these, microscopy was negative in ten cases and antigen-RDT was negative in eleven. The single sample with a negative LAMP finding but a positive qPCR result came from a patient recently treated for low density *P. falciparum* infection[9].

Whole blood and dried blood spots (DBS) were analyzed to detect malaria using LAMP assay. The primers were designed to target the 18S rRNA. The detection limit of *P. falciparum* from whole blood was 25 parasite/ml, while the detection sensitivity of DBS was 50-100 parasite/ml. The DBS could preserve the 18S rRNA for up to five months. The assay achieved 97.0% sensitivity and 99.1% specificity for the identification of all species from asymptomatic individuals. These results indicated that LAMP assay was applicable for detection of malaria in countries with limited economic resources[10]. Also using DBS a total of 911 samples were tested for diagnosing *P. falciparum* infection in Ghana, Uganda, and Tanzania. The primers for PCR and LAMP assays were designed to target 18S rDNA, and the *Pfhrp*2/3 genes. True deletions of *Pfhrp*2/3 gene were verified under the following conditions: microscopy-positive samples, samples tested positive for 18S rDNA PCR, positive results for merozoite surface protein genes using LAMP or PCR, or qPCR detecting more than 5 parasites/µl. No genetic deletions were found in the Ghana samples; however, deletions were detected in Tanzania and Uganda[11].

For diagnosis of *P. vivax* and other *Plasmodium* spp. from 559 febrile patients, two kits were used; the Loopamp Malaria pan detection kit and the Loopamp Malaria *Pv* detection kit. The former yielded 87.7% sensitivity and 94.4% specificity, demonstrating a good strength of agreement with PCR assay. The latter detection kit exhibited 84.4% sensitivity and 92.4% specificity showing substantial level of agreement with PCR assay[12]. The RDTs false negative results were analyzed before and after the implementation of LAMP within the framework of the malaria diagnostic approach. Cases of *P. falciparum* infections that tested negative on RDTs were further examined for potential *Pfhrp*2 or *Pfhrp*3 gene deletions, and sequencing of exon 2 sequences was performed to detect genetic variation that might hinder *Pfhrp*2 identification. From the beginning of the year 2006 to the end of 2018, the prevalence of false-negative *P. falciparum* RDT was low, occurring in 3 out of 446 samples. No cases were observed between the years 2006 and 2016, a period when malaria detection relied solely on microscopic examination and RDTs. However, from 2017 to 2018, when molecular diagnostic methods were introduced for malaria screening, the prevalence increased to 3 out of 73 samples. The analysis found no evidence of *Pfhrp*2/3 gene deletions or significant mutation in the repeated epitopes that could account for the false-negative RDT findings[13].

A total of 71 malaria-positive blood specimens confirmed by microscopy were gathered as DBS. DNA purification was followed by nested PCR and SYBR green I-based LAMP technique targeting the 18S rRNA gene. The changes in color of the LAMP amplicons were visually assessed without specialized equipment. The LAMP method showed a detection sensitivity of 10 copies/µl, outperforming nested PCR, which detected 100 copies/µl. Among the 71 *P. knowlesi*-positive specimens, LAMP successfully identified 69. It showed superior sensitivity compared to the nested PCR. The SYBR green I-based LAMP assay exhibited 97.1% sensitivity and 100% specificity. By integrating SYBR green I into the inner portion of the tube cap, the results could be directly observed after amplification without uncapping. Positive samples became green immediately, while negative samples continued to be orange[14].

Researchers analyzed the diagnostic accuracy of one step technique utilizing reverse transcription LAMP (RT-LAMP) from whole blood for identifying *P. falciparum* malaria in seemingly healthy 256 blood donors and neonates presenting with fever. Identification of malaria parasite presence was by microscopic examination, antigen detection, rapid RDTs, and RT-LAMP. Thirty-six out of the 256 blood donors showed positivity by light microscopy, RDT had positivity of 38, and RT-LAMP detected 78 specimens. In 279 febrile neonates, infection was only identified by light microscopy and RT-LAMP and recorded positive rates of 8.6% for microscopic examination and 12.2% for RT-LAMP. Neonates had lower parasite densities than blood donors. This study highlighted the potential of whole blood RT-LAMP as a valuable pre-screening method for blood donors and for diagnosing malaria in neonates, helping in the prevention of *P. falciparum* consequences[15].

New LAMP primers targeted the cytochrome oxidase I (*cox1*) gene, erythrocyte membrane protein 1 (EMP1), *Pv*r47 for *P. vivax*, and *Pf*r364 for *P. falciparum* were designed. The detection limit for the LAMP assays ranged from 2.4 to 3.7 parasites/µl of whole blood. For detection of *P. vivax*, the LAMP targeted *cox*1 demonstrated superior efficacy than the *Pv*r47 target, but in *P. falciparum*, the most specific was the *Pf*r364[16]. An assay targeting a segment of mitochondrial DNA (mtDNA) across *Plasmodium* spp., including the five human-infecting species, was developed. Two assays targeting 18S rDNA of *P. ovale* and *P. vivax* were also designed. The assays' sensitivity was evaluated based on comparison with nested PCR using specified concentrations of plasmids carrying the target sequences. The detection limit was 102 copies for the mitochondrial DNA. The results demonstrated that 10^2 and 10^3 copies of 18S rDNA could be identified from *Plasmodium* spp., *P. vivax* and *P. ovale*, respectively. The pan malaria mtDNA LAMP assay had 98.48-100% sensitivity and 90.75-100% specificity in comparison with nested PCR technique. In *P. vivax*, 18S rDNA LAMP assay showed (95.85-100%) sensitivity and (98.1-100%) specificity, while in *P. ovale*, sensitivity ranged from 90.76-99.96%; and specificity was between 98.34-100%. The detection limits of the three LAMP assays were lower than PCR[17].

The *Pfkelch13* C580Y mutation was recognized as a diagnostic marker for detecting artemisinin resistance. The study was designed to validate the LAMP technique for identifying the C580Y mutation

by inserting a mismatched single nucleotide at the FIP primer 3' end. The optimal conditions of the assay were 56°C for 45 min, and the results were analyzed using gel electrophoresis and HNB. A total of 120 *P. falciparum* DBS specimens were subjected to LAMP assay. Comparison of the LAMP technique outcomes with genetic sequencing of the clinical specimens showed complete agreement in identifying the C580Y mutation. The sensitivity and specificity of the LAMP assay were both 100%. The LAMP assay was shown to be a fast, and effective method for diagnosing artemisinin resistance[18]. The LAMP assay tested 400 *Anopheles* mosquitoes, with multiplex nested PCR used as a comparison reference method. Six out of 400 mosquito samples tested positive for *P. vivax*. Neither *P. falciparum* nor mixed infections was identified in the study. The results concluded that LAMP could be beneficial in detection of malaria parasites[19].

Puri and co-workers[20] invented a portable LAMP device for malaria detection at point-of-care, especially for *P. falciparum*. The device was provided with a fluorescence readout unit and could be managed using a mobile application. The assay's performance was assessed on fresh and stored clinical isolates of *P. falciparum*. The LAMP assay using *P. falciparum* (3D7), executed on the device to evaluate the limit of detection, revealed 98.89% sensitivity and 100% specificity. Another well-conducted investigation compared the performance of various diagnostic techniques in diagnosis of *Plasmodium* spp. The blood samples of febrile and afebrile cases were analyzed using microscopic examination, RDT, nested PCR and PURE-LAMP. The PURE-LAMP, rapid diagnostic tests and microscopy showed sensitivities of 100%, 85.4% and 49.4% respectively. All techniques yielded specificities over 99%[21]. Moreover, Ivarsson and coworkers[22] compared 2 LAMP kits (Alethia Illumigene Malaria kit and HumaTurbLoopamp Malaria Pan Detection (PDT) kit) for detection of *Plasmodium* spp. in 133 blood specimens. As a result, 41 samples tested positive by qPCR. The HumaTurbLoopamp™ Malaria PDT kit showed 100% concordance with qPCR, with very high sensitivity and 100% specificity. The Alethia® Illumigene Malaria kit yielded 90.24% sensitivity and 95.65% specificity based on comparison with qPCR.

In a recent study by Ahuja[23] only seven samples out of 92 microscopy-negative samples tested positive for malarial DNA using the LAMP assay, while twelve samples tested positive using the RDT. Eight of these 12 samples were from patients with recent malaria infection; however, the other four were false positive. The PPV was 79.7% for the RDT and 89.2% for the LAMP assay. The NPV was 87.9% and 100% respectively. Findings demonstrated that the LAMP technique detected all microscopy-positive cases, indicating perfect concordance with microscopy in these samples. The LAMP couldn't differentiate between malaria species and couldn't detect mixed malaria. Species specific LAMP assays were developed targeting seven malaria species and evaluated against microscopy, qPCR, and ELISA. The LAMP assay for *P. falciparum* identified all asymptomatic samples with a detection limit exceeding 80 genomic copies/µl of the extracted specimen. It had 95.6% sensitivity and 100% specificity. The recorded LAMP sensitivity was higher compared to microscopy and ELISA which had 52.7% and 67.3% sensitivities respectively. Nine samples tested positive for *P. malariae*, revealing coinfections with *P. falciparum*. These results showed that LAMP is more sensitive than microscopy and ELISA[24].

An effective procedure was developed to purify DNA from whole blood samples using two-reagent purification-free protocol utilizing microfluidic cartridges with a piezo pump. This innovative cartridge consisted of a mixing chamber and metering system to dispense a pre-defined volume of the specimen working lysis mixture into four disparate chambers, each having a reaction mixture. Real-time fluorescent LAMP assay demonstrated a sensitivity around 0.42 parasites/µl of whole blood, proving that it is an excellent technique for diagnosing asymptomatic malaria carriers. These results also highlighted that the DNA purification-free protocol for specimen processing is optimized for qPCR assay[25].

Using 200 samples from malaria-endemic areas, 67 tested positives for malaria by microscopy 98 demonstrated positivity using RDT, while 112 tested positive by the LAMP assay. The enhanced detection capability of the LAMP assay was confirmed by detection of 66 out of 67 positive samples by microscopic examination, showing 98% sensitivity when compared to microscopy. Considering microscopic examination as the reference method the RDT detected 62 out of 67 microscopy-positive samples, with 92.5% sensitivity. The findings demonstrated that the colorimetric LAMP had superior sensitivity than microscopy and RDTs. These results suggest that the LAMP assay offers greater sensitivity than RDT in detecting cases confirmed by microscopy[26]. A novel hairpin-mediated amplification (HMA) technique for the detection of 4 *Plasmodium* spp. including *P. vivax*, *P. falciparum*, *P. ovale*, and *P. malariae* was developed. This method, integrated with LFD, offered effective point-of-care testing in settings with limited resources. The detection limit of HMA-LFD was nearly 5 copies/µl. The consistency between HMA-LFD and qPCR was around 96.15%, revealing that the HMA-LFD method is a reliable maneuver[27].

On testing malaria cases by microscopic examination, immunochromatographic test (ICT), and molecular assays, *P. vivax* showed positivity of 84% using ICT and 100% with both PCR and LAMP assays. For *P. falciparum*, ICT revealed 92.7% positivity, while both PCR and LAMP assays yielded 100% positivity. This study elucidated that both PCR and LAMP assays possess high specificity and PPV of 100%, as well as comparable sensitivity to other diagnostic procedures such as ICT and microscopy[28]. The most common genes associated with *P. falciparum* drug resistance are the *P. falciparum* chloroquine resistance transporter gene (*Pf*CRT), the *P. falciparum* multidrug resistance gene 1

(*Pf*MDR1), and the *P. falciparum* Kelch protein *K13* gene (*Pf*Kelch13). Polymorphisms in these genes serve as molecular markers for detecting drug-resistant strains. While nucleic acid amplification tests combined with DNA sequencing are effective methods for detection of these polymorphisms, their need for sophisticated instruments can limit their use in resource-limited areas. Now, the integration of isothermal amplification with clustered regularly interspaced short palindromic repeats (CRISPR)-based detection has revealed optimistic potential for mutation identification at the nucleic acid level. The LAMP-CRISPR systems provide particular robust and straightforward detection capabilities suitable for deployment in remote and resource-limited settings. This study aimed to develop a novel diagnostic approach using LAMP targeting specific genes to identify drug-resistant *P. falciparum* strains. The methodology included sequence analysis of the *P. falciparum* genome, LAMP primer design, and CRISPR target prediction. The designed primers effectively identified polymorphisms linked to drug resistance in *Pf*CRT, *Pf*MDR1, and *Pf*Kelch13 genes[29].

The *Pf*r364 multi-copy repeats of *P. falciparum* were selected as a target for LAMP assay performance which was evaluated in blood samples and saliva and compared to nested PCR. The 18S RNA gene was the target in both assays. An HNB metal indicator was used, and the HNB-LAMP assay showed greater sensitivity with a detection limit as few as one parasite and rapid amplification within fifteen minutes[30]. A combination of a microfluidic chip with LAMP assay (on chip LAMP) was used for identification of 5 zoonotic parasites: *T. gondii*, *C. hominis, C. parvum, T. solium,* and *C. sinensis*. The results showed 98.08 % sensitivity and 97.59% specificity, in comparison with conventional biological techniques[31]. Moreover, the efficacy of the fluorescent-based (SYTO and SYBR green) LAMP, and colorimetric-based LAMP assays were compared to diagnose *P. falciparum*. Simple extraction method was used. The sensitivity of SYTO-LAMP was 0.00001%, while SYBR Green-LAMP detection limit was 0.0001% based on comparison with the sensitivity of colorimetric-LAMP which was 0.01%[32].

A newly designed primer set was used to improve the performance of a colorimetric LAMP. The LAMP assay targeted the A-type 18S rRNA gene of *P. falciparum* and compared it to PCR. The new set of primers achieved an optimal reaction temperature of 65°C for 30 min, remarkably reducing the time required, compared to previous LAMP procedures which needed 45 to 60 min. The LAMP assay detected as low as 0.21 parasites/µl, which is 1,000 times more sensitive than preceding primer sets. Under reaction optimization, the novel primer sets exhibited 100% sensitivity, specificity and accuracy using DBS[33]. Several combinations of recombinase polymerase amplification primers and probes were developed using *P. malariae* genomic sequences. LAMP assay targeted 18S rRNA gene was evaluated, and the product of amplification was visualized using lateral strips. The assay demonstrated 100% sensitivity and lowest detection limit of 10 copies/µl[34].

In six LAMP assays, targeting genus and specific species of malaria, a new simple extraction maneuver was implemented besides the traditional methods to refine the process. Both column-based and saline extraction methods were assessed. An advanced reaction control (dual-LAMP-RC) assay was deliberated. This control assay confirmed that the resulting reactions are as expected. The study also evaluated lyophilized reagents, which can disentangle the process by eliminating the necessity for cold storage. The dual-LAMP assays results were compared with nested-multiplex malaria PCR interpretations. No cross-reactivity occurred with other parasites in dual-LAMP assay, so it was considered highly specific. The column extraction method had a detection threshold of 1.22 parasites/µl versus the saline extraction method which had detection sensitivity of 5.82 parasites/µL. The six dual-LAMP assays had sensitivity and specificity around 100%. The application of lyophilized reagents yielded results that were concordant with the reference procedure[35].

An innovative pipette-assisted amplification of isothermal probes was validated for malaria diagnosis. This method, which did not require microfluidics, detected *Plasmodium* 18S rRNA with tailed probes in an adapted Pasteur pipette. The attached probes were joined to create a template for amplification of an isothermal probe with a set of primers, eliminating the need for DNA extraction and reverse transcription. The technique was evaluated using cultured *Plasmodium* spp. and assessed against qPCR and RT-LAMP in patient's blood specimens. The entire procedure took 60-80 min with reduced manual effort, requiring only a Pasteur pipette and a water incubator. The method demonstrated a detection sensitivity of 1.28×10^{-4} parasites/µl, with 100% specificity[36].

Another study aimed at optimizing the LAMP assay to identify single nucleotide variations in the chloroquine resistance gene of *P. vivax*. A total of 88 *P. vivax*-positive specimens were gathered. The *Pv*mdr1 gene was extracted and amplified from all the cases and then subjected to sequencing. The resulting sequences were examined to detect single nucleotide variations. The assay showed 100% sensitivity and specificity in identifying single nucleotide variations in the analyzed gene. The detection threshold was 0.9 copies/µl, and the minimum DNA template level identified was 1.5 ng/µl. The detected frequency of single nucleotide variations in the *P. vivax mdr1* gene suggested the early emergence of *P. vivax*. resistance to chloroquine[37].

In fact, *P. falciparum* infections pose a risk to military personnel sent to regions with high malaria prevalence. While stationed, cases of malaria were diagnosed using microscopy and RDT. Afterward, malachite green LAMP and photo-enhanced PCR techniques were applied for malaria diagnosis. ELISA was used to detect IgG antibodies targeting the C-terminal 19-kDa region of the merozoite surface protein 1 in *P. falciparum*.

The malaria prevalence during assignment was 33.33%, and 4 cases were identified after assignment: two cases of *P. falciparum*, one of *P. ovale*, and one of *Plasmodium* spp. Serological surveillance indicated a 31.96% in peacekeepers, reflecting high exposure to *P. falciparum* during missions. These results highlighted the importance of active surveillance in military groups to prevent the emergence of new *Plasmodium* spp. from areas where malaria is endemic[38].

***Babesia* spp.**

A LAMP study emphasized significant progress in the detection of canine babesiosis caused by *B. gibsoni*. The LAMP assay was innovated using primers amplifying the hypervariable region of the 18S rRNA sequences. The test showed excellent specificity and did not exhibit cross-reactivity with other parasites. It detected as few as 12 pg of DNA, and this method is about 10-fold higher sensitive than nested PCR. Of the 75 samples subjected to these tests, 43 samples were positive by LAMP while nested PCR identified 37 specimens and microscopy detected 23 samples[39]. Various diagnostic techniques: blood smears, nested PCR, and LAMP were compared. The results showed that LAMP could offer an alternative approach for diagnosis of bovine babesiosis. The assay could detect 47.62% of cases, performing better than blood smears, that identified only 19% of cases, but was still slightly lower than nested PCR, that had 52.38% sensitivity. The positive detection in *Rhipicephalus annulatus* (Boophilus) reinforced the study's relevance in broader understanding of babesiosis epidemiology in Egypt[40].

A user-friendly molecular technique that employed low-cost equipment and combined cross-priming amplification with a vertical flow (CPA-VF) strip was developed for quick *B. motasi* diagnosis. The target of the assay was the 18S rRNA gene with analytical sensitivity of 50 fg. No cross-reactivity was observed with other *Babesia* spp. infecting sheep or humans. When compared to microscopic examination and nested PCR along with gene sequencing, the CPA-VF had 95.2% sensitivity and 95.8% specificity, while qPCR showed 95% sensitivity and 97.9% specificity. These findings suggest that the CPA-VF is a fast, precise, and cheap procedure for detecting *B. motasi*[41].

A LAMP assay for diagnosing *B. bigemina* and detection of amplicon products by hydroxynaphtol blue was validated. The *ama-1* gene was the target in the test and the reaction conditions were standardized. The optimal results were obtained at 63°C within one hour. The test detected as low as 0.00000001% parasitemia, and no cross reaction occurred with DNA of other parasites. This procedure was also capable of amplifying DNA from ten strains of *B. bigemina* across 3 various countries. This study concluded that LAMP assay is a robust and cost-effective procedure for diagnosis of *B. bigemina*[42]. The thermostability of LAMP assay for detection of *B. bovis* was evaluated. The results demonstrated that LAMP assay has good thermostability and that the DNA could persist for 72 h after initial preparation. Variable light sources including LED, neon, and UV lights were assessed as visualization methods in detection of LAMP amplification products and were compared with two conventional PCR agarose gel electrophoresis (PCR-AGE). The usage of neon was 10-fold greater in sensitivity than the PCR-AGE, while the performance of LED and UV rays was 1,000 times more sensitive. The LAMP assay demonstrated high specificity with no cross-reactivity with other DNA[43].

Theileria annulata

Authors assessed the performance of LAMP assay in detection of *T. annulata* based on comparison with conventional cytochrome b PCR and microscopy. LAMP assay sensitivity and specificity were 78.7% and 87.5% respectively, while its PPV was 98.4% and NPV was 29.1%. Conventional PCR showed 70% sensitivity and 75% specificity[44].

Toxoplasma gondii

The specificity and detection limit of nested PCR and LAMP techniques targeting the 529-bp and B1 genes were performed for the identification of *T. gondii* DNA in blood from infected mice. RE-LAMP detected 1 fg DNA, B1-LAMP detection limit was 100 fg DNA, RE-nested PCR identified as low as 1 picogram (pg) DNA and B1-nested PCR analytical sensitivity was 10 pg DNA. Both the LAMP and nested PCR assays had specificities of 100%. The results revealed that the LAMP technique is extremely sensitive for diagnosis of *T. gondii*[45]. A total of 34 surface water specimens were passed through membrane filters, and subjected to DNA extraction. PCR and LAMP methods were employed to identify *Acanthamoeba* spp. and *T. gondii*. Of the 34 specimens, 30 were positive for *Acanthamoeba* spp. and 2 for *Toxoplasma* oocysts. Two samples tested positive for both parasites. The findings concluded that water supplies examined in Iran were contaminated with *Acanthamoeba* spp. and *T. gondii*. The study indicated that *Acanthamoeba* could be involved in the waterborne spread of *Toxoplasma* in the research area[46].

In another study, a total of 71 air specimens obtained from diverse environments were gathered using gelatine filters. The recovered substance was investigated by real-time PCR and LAMP techniques targeting the B1 gene for identification of *T. gondii*. The DNA was identified in 2 air specimens, proved by both LAMP and real-time PCR assays. Sample DNA genotyping at the SAG2 locus detected them as SAG2 type I. Microscopy confirmed *T. gondii* oocysts identification in one positive air sample[47]. Also, in another study in which the LAMP primers were designed to target the 529-bp fragment for diagnosis of toxoplasmosis, the assay was able to recognize as low as a single tachyzoite or 10 copies of a recombinant plasmid. No cross-reactivity occurred with DNA of different parasites. Two hundred human blood specimens were analyzed using both the LAMP assay and conventional PCR. The LAMP method detected 14

positive cases, whereas only 5 cases were identified by PCR. The LAMP technique was carried out at a constant temperature of 64°C, with amplification time ranging from 35-60 min offering rapid amplification, ease of use, and simple detection. It was therefore considered an excellent option for the clinical diagnosis of acute toxoplasmosis, particularly in resource-limited settings[48].

For validation of the diagnostic accuracy of a commercially available LAMP kit, it was compared with real-time PCR method. Amniotic fluid and both negative and spiked plasma samples, with varying concentrations of live *T. gondii* tachyzoites, were tested. It was then evaluated using a cohort of 11 amniotic fluids, 5 placental, and 32 blood clinical specimens stored at -20°C. For the placental and blood specimens, a preliminary step was applied that did not fully adhere to the manufacturer's guidelines. Simplicity and adherence to standard operating procedures were assessed. However, LAMP technique showed lower sensitivity than PCR at extremely low parasite concentrations; both techniques provided the same qualitative results and, in some cases, quantitative results, especially for amniotic fluid specimens[49]. In collected DBS specimens from mice challenged with *Toxoplasma* ME-49 strain at one week and eight weeks after-infection, both LAMP and conventional PCR methods were used to amplify the 529-bp repeat element. In the first week post-infection, 18 out of 20 DBS specimens were positive by LAMP versus 16 in conventional PCR. In the late stage of infection, neither LAMP nor PCR could detect parasite DNA[50].

A LAMP-LFD procedure targeting 529-bp of *T. gondii* was developed. This innovative portable tool is versatile, robust, simple, and ensures high diagnostic sensitivity while decreasing the risk of aerosol contamination. The detection threshold was 1 fg DNA, and no cross-reaction with DNA of other parasites occurred. Blood specimens gathered from 318 stray dogs and cats were tested by LAMP. The tool identified *T. gondii* DNA in 4.76 % of stray cats and 4.69% of dogs. These results concluded that the technique is applicable for *T. gondii* detection in primary health care facilities and remote environments[51].

A study was conducted involving 117 cases of ocular toxoplasmosis and 200 control participants to validate the LAMP procedure. Blood samples were analyzed by uracil DNA glycosylase-integrated LAMP (UDG-LAMP) and qPCR methods, focusing on the 529-bp and B1 targets. The detection threshold for qPCR targeting 529-bp was 0.1 fg DNA, whereas for B1 it was 1 fg DNA. For UDG-LAMP, the detection sensitivity was 1 fg for 529-bp, and 100 fg for B1 gene. Using qPCR, 18 patients were positive for 529-bp, and 16 patients were positive for B1. UDG-LAMP identified 15 positive cases for 529-bp and 14 positive cases for B1. Overall, qPCR proved to be more sensitive than UDG-LAMP for diagnosing *T. gondii* DNA in peripheral blood specimens from cases with clinical diagnosis of toxoplasmic chorioretinitis. Additionally, 529-bp demonstrated a superior detection rate for identifying ocular toxoplasmosis in blood specimens compared to the B1 gene. The 529-bp showed a higher detection rate for diagnosing ocular toxoplasmosis in blood samples compared to the B1 gene[52].

Samples of Me-49 infected mice brains were analyzed using a membrane-based DNA extraction technique. Fluorescence-based and visually interpreted LAMP amplicons were evaluated and compared. The ideal reaction mixture was optimized by incorporating the protective agent trehalose and adjusting the levels of $Mg2^{+}$ and dNTPs. To minimize aerosol contamination, paraffin and lyophilization methods were employed, and anti-contamination guidelines were followed. Both quantitative LAMP and visual LAMP had a detection limit of 92 copies/µl. The diagnostic performance of both methods was evaluated using 200 feline fecal specimens. Compared to qPCR, quantitative LAMP showed 100% sensitivity and 100 % specificity, while visual LAMP showed 100% specificity and 80% sensitivity. The study concluded that quantitative and visual LAMP assays were rapid, straightforward, and required minimal sample processing[53]. In another study, a visual LAMP technique targeting the B1 gene for identifying *T. gondii* oocysts in cat feces was validated. The results of amplification were interpreted visually by color change. The detection sensitivity of LAMP was 10 copies/µL which was ten times greater than PCR and no cross reactivity with DNA of other parasites. The LAMP assay identified a single oocyst in 83.3% of specimens while PCR detected only 50%[54].

Brain homogenates were collected from mice at eight weeks after-infection with *Toxoplasma* ME-49 strain. LAMP assay findings were analyzed in comparison with conventional PCR and histopathological data. The LAMP technique recognized DNA in 18 out of 26 samples, meanwhile conventional PCR identified only 15 samples[55]. The new innovative LAMP DNA chromatography technique was designed to amplify 529-bp fragments of *T. gondii* DNA in patients suspected to have toxoplasmosis. The LAMP DNA chromatography method demonstrated 68.2% sensitivity and 100% specificity in comparison with conventional LAMP which had 63.6% sensitivity and 100% specificity. The nested PCR revealed 45.4% sensitivity and 100% specificity[56]. A total of 100 wild rats were gathered from 3 different regions in Surabaya. *T. gondii* LAMP assay detected 30% positivity in brain tissue. The prevalence was higher in male rats than female rats. The prevalence varied according to location, 41.9% of rats from housing areas were detected positive, the positivity in traditional markets was 33.3% and 22.6% of rats from dense settlements were positive[57].

Neospora caninum

Six primers were developed to target the Nc-5 gene of *N. caninum* in a LAMP assay in comparison with semi-nested PCR assay. In LAMP,

the amplification was observed in 30 min with an optimal temperature of 63°C, and a low detection limit of 1 fg which was 10 folds greater than PCR. The LAMP assay showed 39 % positivity while semi-nested PCR demonstrated 36% positivity. The study highlighted the advantages of LAMP technique in *N. caninum* diagnosis[58]. A LAMP assay for *N. caninum* was introduced, and ten-fold serial dilutions of tachyzoites were prepared to detect the sensitivity in comparison with PCR. The LAMP results were evaluated visually based on color change. The field applicability of the LAMP procedure was evaluated in 396 blood specimens, 115 fecal dog samples, and a single placenta from a female calf. No DNA amplification from other organisms was observed, and the detection sensitivity was one genome in both LAMP and qPCR. Both techniques had 3.8% positivity in blood samples. LAMP and qPCR identified 9 and 5 positive cases out of 115 fecal specimens, respectively. Both assays confirmed the presence of infection in the placenta. There was a great concordance between LAMP and qPCR in tested blood specimens and the agreement in fecal specimens was substantial[59].

***Cryptosporidium* spp.**

Research was conducted to investigate the molecular identification of *Cryptosporidium* spp. in environmental water resources. S-adenosyl-L-methionine synthetase (SAM) was the target gene in LAMP assay while nested PCR targeted small subunit rRNA gene. A total of 240 and 180 environmental samples were taken from 20 and 25 different locations respectively. Results showed that 42% and 38.3% out of the 240, and 41.1% and 38.8% out of 180 environmental samples tested positive by LAMP assay and nested PCR, respectively[60]. A total of 168 surface water samples were tested using primers targeting the SAM gene in LAMP assay for detection of *Cryptosporidium* spp. oocysts. Overall, 69 of 168 specimens showed positivity for *Cryptosporidium* spp. oocysts over eleven months examination period. This Turkish study compared the presence of oocysts in Tersakan and Yesilirmak rivers, and it showed high rates of contamination in the former. The study demonstrated that LAMP assay was one of the most beneficial molecular techniques that did not require sophisticated instruments[61].

The LFD LAMP technique was innovated for detection of *Cryptosporidium* spp. The primers targeted SAM-1 gene and stem primers were used to accelerate the reaction. The stem LFD LAMP assay demonstrated low detection sensitivity of 10 oocysts/ml outperforming the SAM-1 LAMP assay and nested PCR, which had detection limits of 100 oocysts/ml. The stem LFD LAMP showed high positivity and high specificity[62]. A study was conducted to evaluate the zoonotic risk between animals and their farmers or owners. DNA specimens obtained from the feces of 17 cattle and 38 water buffaloes, previously diagnosed with *C. parvum* by Kinyoun acid-fast stain in an earlier study, were tested by LAMP method. Acid-fast stain findings and LAMP results were evaluated in comparison to PCR results from both farmers and owners to assess the potential zoonotic transmission of *C. parvum*. The LAMP assay revealed that 41% of cattle and 76% of water buffaloes tested positive. Comparison with previous PCR results indicated potential zoonotic transmission of *C. parvum*. Eight farmers who tested positive for *C. parvum* by PCR and had water buffaloes that were positive in the LAMP assay were found to be infected. Just a single farmer, who had cattle, was PCR-positive for *Cryptosporidium* spp., but negative in the LAMP technique, suggesting that species other than parvum could cause infection in both the farmer and the animal[63].

Fluorescence-based and lateral flow strip recombinase polymerase amplification (LFS RPA) targeting the 18s rRNA gene for *Cryptosporidium* spp. was developed. Fluorescence-based recombinase polymerase amplification (real-time RPA) assay and LFS RPA displayed DNA amplification within 20 min. When a metal bath was used for incubation at 42°C, LFS RPA results were achieved within 5 min while results of the real-time RPA could be detected at 39°C. The two RPA assays demonstrated 100 % sensitivity and 91.67 % specificity[64]. The investigators compared the analytical sensitivity of rapid colorimetric LAMP test and real-time PCR in detection of *Cryptosporidium* spp. from feces of 127 calves. Both assays targeted the 18S rRNA gene. The analytical sensitivity of LAMP was one copy plasmid/reaction. The LAMP results showed 100% sensitivity and 97.4% specificity[65].

***Leishmania* spp.**

Four primers were designed to target the 18S rRNA gene conserved region. Malachite green (MG) was used for visualization of amplification products at the optimal time of 75 min. *L. siamensis* promastigotes were combined with saliva or blood to mimic crude specimens. Samples were collected from cutaneous (CL) and visceral leishmaniasis (VL) patients. All specimens were directed to simple boiling for 10 min then added directly to the LAMP reaction. The detection sensitivity of *L. siamensis* was 10^3 parasites/ml or 2.5 parasites/tube. The usage of whole blood, tissue biopsies and saliva did not negatively impact the detection sensitivity. Furthermore, this LAMP assay was capable of detecting DNA from various *Leishmania* spp.[66]. A study validated the LAMP test for diagnosing *L. infantum* in dogs. Kinetoplast minicircle DNA (mkDNA) sequences of *L. infantum* was the target of the assay. Conjunctival swab samples were received from one hundred and eleven dogs in an endemic region and 33 dogs from a region not endemic for VL. The detection limit of the LAMP assay was 1 fg of *L. infantum* DNA which was 10-fold greater than conventional PCR test showing high specificity of 97%. The results demonstrated 61.3% positivity by LAMP, 58.6% by PCR, 40.5% by ELISA and 10.8% by microscopy[67].

A study aimed to validate three different LAMP assays for diagnosis of *Leishmania* spp. DNA in human blood or tissue samples and compared to qRT-kDNA

PCR. Two LAMP assays targeting the internal transcribed spacer 1 (*ITS1*) gene, which is conserved among various *Leishmania* spp., were developed. The third LAMP system was based on a novel repetitive region of the *Leishmania* genome. The LAMP systems detection limit was 0.1 pg of DNA from different *Leishmania* spp. Addition of SYTO 16 to LAMP assay allowed continuous real-time monitoring of the amplification process, so it was superior to traditional LAMP assays, which only revealed results after the reaction was ended. The validity of the LAMP test in diagnosis of *Leishmania* DNA from dried blood samples, was compared to that of qRT-kDNA PCR. Results showed that LAMP assay was a reliable and robust technique in diagnosis of VL in endemic regions[68].

A study focused on assessing two molecular methods including pre-addition of malachite green to mkDNA PCR, for distinguishing *L. martiniquensis* in sand fly vectors, using ITS1 PCR as a reference standard. PCR-mkDNA had surpassed sensitivity, as it could detect 1 promastigote per gut, making it ten-fold more sensitive than MG-LAMP and PCR-ITS1 and it was also highly specific because no cross-reaction occurred with other parasites. Screening of 380 sand flies in Thailand using PCR-mkDNA was applied, and no *Leishmania* DNA was detected[69]. A study for detecting *Leishmania* spp. infections using LAMP assay, particularly targeting *L. donovani* kDNA, showed outstanding sensitivity and specificity. The sensitivity results for VL in blood samples were 96.9% and the bone marrow aspirates were 100%. Sensitivity of post kala-azar dermal leishmaniasis (PKDL) in tissue biopsies was 97% while in tissue biopsies for CL, it was 80%. The specificity of the assay was 100% in both blood and tissue biopsies. The assay detected *L. donovani* DNA as small as 1 fg within 30 min, and the assay was also used for evaluation of cure. The DNA in 2 out of 20 VL blood samples and 2 out of 21 PKDL tissue biopsies post-treatment was detected. Noticeably, two patients (1 VL and 1 PKDL) who had received treatment tested positive for parasite DNA indicating relapse[70].

A pan-*Leishmania* LAMP test was invented for the diagnosis of CL and VL. A set of primers targeting the 18S rRNA and the conserved region of mkDNA was designed. LAMP assay was assessed for diagnosing CL, in comparison to combined reference of microscopic examination and/or culture. The LAMP assay was also evaluated on whole blood, peripheral blood mononuclear cells, and buffy coat samples using microscopic examination of splenic or bone marrow aspirates as a gold standard. Results demonstrated that sensitivity and specificity for CL were 95% and 86%, respectively while for VL sensitivity and specificity were 92% and 100%, respectively[71]. In another assessment of the diagnostic performance of a LAMP assay, the 18S rRNA gene was the target in detection of 6 New World *Leishmania* spp. Fifty sandfly specimens and fifty direct smears from the CL outbreak were tested by LAMP by specifically amplifying *Leishmania* DNA; and the limit of detection was set at 1×10^{-2} equivalent parasites per ml. A 100% sensitivity was observed in both direct smears and sandfly specimens. Specificity was 90.9% in direct smears compared to microscopy and 96.8% in sandflies compared to qPCR[72].

The *Leishmania* spp. LAMP Kit was assessed for its ability to diagnose VL in Sudan. A total of 198 individuals suspected of having VL were examined using microscopy of aspirated lymph node specimens (considered the reference standard), a DAT, and RDT based on the rK28 antigen. LAMP was conducted on whole blood and buffy coat peripheral blood that had been prepared using simple boiling and kit extraction methods. Of the 198 VL suspects, 97 were affirmed as infected by microscopic examination of lymph nodes. The rK28 RDT had 98.81% sensitivity and 100% specificity; DAT achieved 88.10% sensitivity and 78.22% specificity. The simple boiling demonstrated 97.65% sensitivity and 99.01% specificity, while kit extraction had 100% sensitivity and 99.01% specificity[73].

A prospective study was conducted in Kabul, focusing on the evaluation of different tests for diagnosing CL. Slit skin samples from suspected patients were examined microscopically, and specimens obtained with a dental broach were analyzed with CL Detect, PCR and Loopamp. The accuracy of the tests was assessed against a composite of microscopy and PCR as a standard reference. Results demonstrated that CL Detect had 65.4% sensitivity and 100% specificity. LAMP showed 87.6% sensitivity and 70.6% specificity when evaluated in Kabul. However, when analyzed at Academic Medical Center, LAMP showed higher efficacy with 92.2% sensitivity and 94.1% specificity[74]. Sakiand co-workers[75] tried to overcome the obstacles of PCR in detection of CL. A malachite green LAMP assay using a target of the rRNA gene was performed, while in nested PCR, mkDNA primers were used. The 75 clinical samples in both assays were collected from patients whose infection had already been confirmed microscopically. Both assays successfully identified the presence of the parasite in 100% of the samples. However, LAMP proved to be more reliable, detecting *L. major* down to a level of 10^4 parasites/ml.

A study that aimed at assessing the LAMP technique targeting the K26 *L. donovani* complex antigen-encoding gene. The reference standard for detection of the diagnostic efficacy of the LAMP technique was a combination of parasitological and/or serological tests. The analytical sensitivity of the K26-LAMP assay was 1 fg of *L. infantum* purified DNA based on comparison with PCR targeting mkDNA genes. K26-LAMP assay had 98.2% sensitivity, 98.1% specificity and 98.2% accuracy while kDNA *Leishmania*-PCR test showed 100% sensitivity, 100% specificity and 100% accuracy[76]. The objective of another research was to optimize the LAMP assay designed for *L. amazonensis*, targeting its kDNA minicircle sequence. Results were compared against conventional PCR and qPCR results. The LAMP assay revealed 100% specificity and 89% sensitivity. It surpassed qPCR in sensitivity, being

reproducible for detecting as few as 28 parasites in 50 ng of DNA[77].

A novel approach to closed tube LAMP was applied by integrating a semi-layer technique. A parafilm was incorporated between the SYBR Green I dye and the LAMP reaction mixture to reduce the risk of dye spillage. This method enabled visual detection of results without the need for post-amplification steps. Results could be easily interpreted under both visible and UV light after spinning down the dye. This modification in the assay successfully amplified *Leishmania* spp. DNA, yielding 94.4% sensitivity and 96.9% specificity[78]. The purpose of another study was to assess the diagnostic effectiveness of LAMP assay for diagnosis of canine leishmaniasis (CanL) in comparison to qPCR and three serological tests which were ELISA, indirect fluorescent antibody test (IFAT), and rapid SNAP test. Out of the samples tested, 41 (68.3%) detected positive for at least one diagnostic assay. IFAT emerged as the most reliable serological test, with 100% specificity and 97.2% sensitivity, detecting more positive cases than the other methods. For molecular diagnostics techniques, fine-needle aspiration of lymph nodes as a sampling method proved to be superior. LAMP demonstrated better agreement with qPCR ($\kappa = 0.80$), and statistical significance ($P<0.0001$)[79].

Researchers created a portable LAMP system designed for detecting leishmaniasis. Samples were collected from VL and PKDL patients to validate LAMP assay, and primers included the ITS-1, kDNA, and the 18S rRNA gene. The LAMP technique efficiently amplified tiny amounts of *L. donovani* DNA, from 1 ng down to 100 fg, in just one hour. The LAMP assay exhibited 100% sensitivity and 98% specificity. The results were compared to those obtained from real-time PCR, and LAMP findings exhibited highly significant agreement with PCR. Specimens were collected from non-endemic controls and leprosy cases to test assay specificity. In control samples, the LAMP assay did not identify *L. donovani*, aligning perfectly with PCR results. Also, in leprosy cases with no previous exposure to *Leishmania*, both PCR and the LAMP assays confirmed the absence of *L. donovani*. These findings established the LAMP assay as a simple, and effective device for diagnosing VL and PKDL[80].

A study was conducted on blood and urine specimens from 720 individuals, including household members and adjacent contacts of 276 primary cases of VL and PKDL, who had no symptoms or history of disease, in endemic areas of Bangladesh. All specimens were subjected to qPCR, DAT, ELISA, and LAMP assays. The qPCR identified asymptomatic infection in 1.0%, LAMP in 2.1%, DAT in 3.9%, and ELISA in 3.3%. Only a single participant tested positive in all 4 diagnostic techniques. The concordance between the tests was poor. However, combining the ELISA and DAT identified all participants who tested positive by more than one technique. A moderately strong correlation was found between being a primary case with PKDL, particularly macular PKDL, and testing positive in at least one of the 4 diagnostic methods[81]. A multiplex LAMP technique was used for *L. donovani* detection. The reaction was rapid, and amplification was observed in 15 min. It had a low detection limit of 1 fg and showed 100 % sensitivity for PKDL detection. Also in this research, the authors assessed the validity of the technique for *M. leprae* detection. The analytical sensitivity was 100 fg for *M. leprae*. The assay demonstrated 95% sensitivity for detecting leprosy. All the PKDL and leprosy specimens revealed positivity using both qPCR and LAMP techniques. These results elucidated that multiplex LAMP technique was useful for distinguishing between leprosy and PKDL[82].

Researchers evaluated the diagnostic performance of three methods: the LAMP assay, real-time PCR assay, and ELISA in diagnosis of *Leishmania* spp. using various sampling methods. A total of 80 VL cases, confirmed by the rK39 RDT, and eighty endemic control samples were tested. Peripheral blood, DBS, and urine samples were gathered from all participants. DNA was extracted from all specimens by boiling and kit-based methods. Urine samples were analyzed with ELISA. Post-treatment, urine specimens from forty-one patients were collected twice, at day 30 and day 180, and analyzed using ELISA. The sensitivities of the LAMP on whole blood extracted by boiling was 96.2%, while for the kit it was 95%. On DBS with kit-based extraction, LAMP showed 85% sensitivity. For qPCR, the sensitivities were 93.8% for whole blood and 72.5% for DBS. All molecular methods had 100% specificity. The ELISA showed 97.5% sensitivity and 91.95% specificity, and demonstrated full recovery in all monitored cases by day 180. Overall, the LAMP on whole blood extracted by boiling and ELISA were identified as potential diagnostic procedures for VL[83].

A study aimed to achieve an ultra-sensitive and highly selective one step LAMP assay for detection of *Leishmania* spp. by using a combination of colorimetric and fluorescence identification methods. The assay utilized gold-nanoparticle probe (AuNP-probe) for colorimetric analysis, SYBR safe dye for fluorescence detection of DNA amplification products, and distance-based paper device (dPAD) for visual interpretation. The sensitivities and specificities of the three identification techniques were 95.5%. The assay demonstrated a lower detection threshold of 10^2 parasites/ml which was ten-fold more sensitive than similar studies[84]. Tape-disc LAMP (TD-LAMP) technique was innovated to implement significant advancement in diagnosing CL. The primer sets used in the assay targeted kDNA of *L. tropica*, and the TD-LAMP assay yielded detection limit of 1 fg DNA. The results were outstanding with a sensitivity of 97% for detecting *L. tropica* infections and specificity was 100%[85].

Evaluation of diagnostic assays for VL in Ethiopia, compared rk39 RDT, direct agglutination test (DAT), microscopy, LAMP, and miniature direct-on-blood polymerase chain reaction-nucleic acid lateral flow immunoassay (mini-dbPCR-NALFIA) using qPCR as reference standard. The qPCR results showed

61.28% positivity in suspected cases of VL. The rk39 RDT demonstrated 88.11% sensitivity and 83.33% specificity. DAT had 96.50% sensitivity and 97.96% specificity while microscopy revealed 76.58% sensitivity and 100% specificity. Sensitivity and specificity of LAMP were 94.33% and 97.38% respectively, while sensitivity and specificity of mini-dbPCR-NALFIA were 95.80% and 98.92% respectively[86]. To validate LAMP assay for diagnosing CL, primers were designed targeting the heat shock protein HSP70 gene. The assay detected 100 fg of purified *L. braziliensis* DNA and yielded high specificity. The accuracy of the assay was 92%[87].

Specific primers for LAMP assay were designed to target the kinetoplast 5.8S rRNA of *Leishmania* spp. The DNA of different parasites was used to assess the test specificity. The sensitivity limit was 1 pg/µl without calcein and 1 ng/µl with calcein. The average turbidity peak values without and with calcein were 0.194 and 0.120, respectively, and after adding calcein, there was a delay of 23.6 min in turbidity. The LAMP and qPCR methods were employed to test 67 blood samples from febrile patients, identifying the same two samples as positive. Microscopic examination of bone marrow smears corresponding to these two positive blood samples revealed the presence of *Leishmania* amastigotes[88]. A study was performed on 122 preserved specimens on tape discs from suspected CL cases in Ethiopia. Microscopic examination of skin samples was performed on all patients. DNA was purified from the tapes and tested using PCR and LAMP. The primers were designed to target spliced leader RNA, and kDNA. Diagnostic performance was evaluated by using microscopy as the standard reference method. Microscopy detected 64 out of 122 patients. The PCR tests exhibited 95.3% sensitivity whereas LAMP showed 48.4% sensitivity and 87.9% specificity. The LAMP limit of detection was 100 times lower for *L. donovani* (20 fg/µl) compared to *L. aethiopica* (2 pg/µl)[89].

African trypanosomes

A study for trypanosome species identification involved examination of cattle, goats, and tsetse fly specimens. Samples were tested microscopically and using two molecular techniques: PCR and LAMP. Results showed poor concordance among the diagnostic techniques for detecting trypanosomes. Microscopy detected trypanosomes in 6.1% of animals, PCR in 7.5% and LAMP in 18.6%. LAMP targeting the paraflagellar rod A (*Pf*R-A) gene was found to be more sensitive than PCR targeting ITS region of kDNA for detecting trypanosomes. *T. congolense* and *T. vivax* infection in cattle and goats were correlated with clinical animal African trypanosomiasis[90]. To investigate the effectiveness of LAMP and PCR assays in diagnosis of *T. b. gambiense* IL3253 strain infection in 6 vervet monkeys, daily parasitemia evaluations were recorded over a 180-day period. Weekly collections of blood, saliva, CSF, and urine specimens were used. PCR and LAMP analyses were conducted on all specimens. LAMP detection was notably more sensitive than parasitological procedures and PCR across all specimen types. The effectiveness of LAMP differed, showing the best results in serum, then saliva, and lastly urine specimens. In saliva specimens, LAMP achieved 100% identification between 21 and 77 days post-infection (dpi), while in urine samples, detection was slightly less, but still above 80% between 28 and 91 dpi. However, LAMP failed to identify trypanosomes in saliva after 140 dpi and in urine after 126 dpi. The results of this study highlighted the significance of LAMP for diagnosing African trypanosomiasis in different samples[91].

Hayashida and coworkers[92] introduced significant improvements to the LAMP technique for diagnosis of African trypanosomiasis in remote settings. The first key development involved drying and stabilizing the LAMP reagents in a single tube, with the use of trehalose to extend storage stability at room temperature. The second improvement simplified specimen processing, allowing for direct amplification of nucleic acids from blood specimens lysed with detergent. These advancements made it feasible to diagnose human African trypanosomiasis (HAT) in community health centers or villages in regions affected by the disease, potentially revolutionizing the diagnostic applications for all tropical diseases. A LAMP assay identifying the 5.8S rRNA ITS2 gene of *T. b. gambiense* is available, but it does not address binding sites covering the CCCA (C3A) insertion region (557-560 bps) which further distinguishes *T. b. gambiense* from *T. b. brucei*. A study presented the 5.8S-ITS2-based LAMP method to meet these criteria. The LAMP primer designs, including the C3A four-nucleotide sequence of *T. b. gambiense* at the beginning of the forward outer primer, demonstrated outperforming specificity and sensitivity, detecting as little as 0.1 fg of *T. b. gambiense* DNA[93].

Patients suspected of having *gambiense*-HAT at outpatient clinics in Uganda were first screened using RDT, followed by microscopic examination. If a patient tested positive with the RDT but negative by microscopy, blood specimens were further analyzed by LAMP assay. Patients who tested positive by LAMP were regarded as high-risk cases and were subsequently re-assessed by microscopy. The selection and enhancement of facilities to conduct microscopy and LAMP were based on the results from spatial referencing and profiling of all community health centers in the 7 endemic regions in Uganda. Three centers were advanced to offer LAMP, RDTs, and microscopy, nine were equipped for RDTs and microscopy, and 200 centers were designated for RDT screening. This strategy decreased the median distance that a patient must travel for HAT screening from 23 km to 2.5 km. As a result, nine HAT patients were detected in 2014 and four in 2015[94].

A LAMP dry format for *T. evansi* diagnosis was invented and it detected as few as 1 parasite per assay. Blood samples from 48 camels were tested positive by LAMP, and all samples tested negative by microscopy

showed 89.6% positivity by LAMP assay. Additionally, DNA extracted from 20 random chosen blood specimens was tested by RoTat1.2-PCR, yielding 14 corresponding results, with 6 samples tested positive by dry-LAMP but negative by PCR. Direct blood kappa value for dry-LAMP was 0.4211, revealing moderate concordance with RoTat1.2-PCR. Furthermore, 103 DNA specimens purified from camel blood analyzed with both dry-LAMP and RoTat1.2-PCR, showed 67 concordant results, 31 tested positive by dry-LAMP and negative by PCR, and an additional 5 positive by PCR revealed negativity with dry-LAMP[95]. Repetitive insertion mobile element (RIME) and 5.8S rRNA-ITS2 gene of *T. brucei* were used as a target in LAMP assay. This study assessed sample incubation with detergent, and concluded that the positive cases could increase with the usage of detergent[96].

To evaluate the role of the vector a total of 82 specimens of live tsetse flies were dissected, and mid-guts were examined microscopically for trypanosomes. Both the mid-guts of the live flies and some dead flies underwent a DNA extraction process. The primers of PCR and LAMP assays were designed to target ITS1 region and RIME. Microscopy, and RIME LAMP revealed positivity rates of 96.3% and 50%, respectively. Additionally, ITS1 PCR detected trypanosomes in 58% of flies examined, with 64.4% identified as *T. brucei*, 8.1% as *T. simiae*, 10.3% as *T. vivax*, 66.9% as *T. godfreyi*, and 6.9% as *T. congolense*. Only 10 specimens showed mixed infections[97].

Equine blood samples artificially contaminated with determined amounts of parasites were used to identify the detection threshold of *T. brucei* ssp. The findings were compared with the gold standard for antemortem diagnosis, *T. brucei*-PCR (TBR-PCR). The detection threshold for *T. brucei* spp. in whole blood DNA was 1 parasite/ml for both LAMP and TBR-PCR, with perfect concordance (14/15) observed between the tests at high parasite concentrations (1 x 10^3/ml). The detection sensitivity for LAMP on whole blood was 100 parasites/ml, which decreased to 10 parasites/ml when detergent was added. At high parasite concentrations (1 x 10^3/ml), LAMP performed very well, however, it showed greater variability at lower concentrations. The concordance between tests ranged from weak to moderate, with the highest agreement seen between TBR-PCR and LAMP on whole blood DNA. A prospective observational study was also conducted on working equids in Gambia that fulfilled the clinical criteria for trypanosomiasis. LAMP was assessed in comparison to subsequent TBR-PCR testing. Analysis of 321 whole blood samples was done in field conditions. The agreement between LAMP on whole blood and TBR-PCR was low, however excellent agreement was observed when examining cerebrospinal fluid[98].

Assessment of the performance of LAMP using clinical specimens from *T. b. gambiense* and *T. b. rhodesiense* patients and tested by a semi-automated procedure utilizing a bio-inkjet printer was employed. Test tubes with precision for a dried LAMP assay, referred to as CZC-LAMP was employed. Performance of the CZC-LAMP test maintained its stability when stored at 30°C for up to 180 d. *T. b. gambiense* LAMP had 72% sensitivity, and *T. b. rhodesiense* showed 80% sensitivity. The specificity of the assay was 95% by testing DNA from 116 controls collected from endemic areas. The assay demonstrated 100% sensitivity and specificity using 14 unprocessed blood lysate specimens from *T. b. rhodesiense* patients and controls from endemic areas. The CZC-LAMP procedure, which eliminated the need for a cold chain or complex laboratory facilities, showed significant potential as a point-of-care test for HAT detection in endemic regions[99].

American trypanosomes

In 2017, Besuschio *et al*.[100] developed a hopeful LAMP-based prototype kit to detect *T. cruzi* DNA. This method offered a robust, user-friendly tool for Chagas disease diagnosis. The assay targeting a highly repeated satellite DNA sequence of *T. cruzi* was evaluated. It could detect as low as $1x10^{-2}$ fg/µl for *T. cruzi* DNA and $1x10^{-2}$ parasite equivalents per ml in EDTA-treated blood. The usage of reagents in dried form and calcein fluorescence visualization disentangled the process by allowing results to be viewed directly, implementing affordable, cost-effective diagnosis in limited resources. The prototype revealed high sensitivity and specificity in detection of all six discrete typing units (DTUs) of *T. cruzi*. When compared to qPCR, the LAMP assay matched or exceeded the performance of qPCR's for specific specimens, such as spiked EDTA blood. The kit's accuracy in different Chagas disease phases, held outstanding potential for enhancing disease diagnosis and treatment selection.

The validity of LAMP assay was further authenticated for diagnosing *T. cruzi* in different clinical samples, demonstrating 93% sensitivity and 100% specificity in blood specimens. The LAMP technique and qPCR showed a high degree of agreement with a κ value of 0.92. The Loopamp kit revealed both sensitivity and specificity for identifying *T. cruzi* infection. It was applied to DNA purified from different sample types, including peripheral blood (frozen blood samples preserved with EDTA, blood preserved in guanidine hydrochloride-EDTA solution, blood preserved with DNAgard solution, and DBS) and CSF samples, targeting infections caused by different parasite strains. The kit showed promise as a rapid diagnostic procedure for *T. cruzi* infection in cases of congenital transmission, acute infection, and reactivation of Chagas disease associated with HIV coinfection[101].

Research identifying a highly conserved 231 bp repetitive satellite region across different strains by LAMP assay, proved to be an effective molecular marker for *T. cruzi* diagnosis, successfully detecting DNA of different *T. cruzi* strains. The detection limit for the CL Brener strain was 5 fg, while for the DM28 strain, it was 50 fg. It showed no cross-reactivity with DNA from sixteen helminth species or seven protozoa. LAMP assay, leveraging the highly repetitive satellite

fraction, is proposed as a valuable alternative for *T. cruzi* diagnosis, addressing challenges of existing methods such as analytical sensitivity, velocity, and dependence on advanced tools or expertise. Moreover, the LAMP technique is well-suited for adaptation as a molecular test in resource-limited settings[102].

Other researchers integrated the *T. cruzi*-LAMP assay prototype with a repurposed 3D printer for DNA extraction. The entire procedure needed less than three hours to produce results, with detection threshold ranging from 0.1 to 2 parasites/ml based upon the strain. A total of 25 blood specimens collected from neonates of seropositive mothers were analyzed in a blinded manner. When compared to real-time PCR, the integrated procedure demonstrated strong agreement. Both molecular techniques provided excellent sensitivity and specificity relative to microscopic assessment of congenital Chagas disease. The integrated procedure successfully identified all ten cases of congenital Chagas disease, highlighting its successful potential[103].

A study introduced a straightforward device and detection technique that used paper discs linked to contactless conductivity Chagas disease sensors. The amplification products in paper diagnostic devices were examined using hydroxynaphthol blue and fluorescence methods. The results revealed that the analytical device is effective for amplifying DNA templates of *T. cruzi*. The conductivity signals were analyzed following integration of the C4D detector. Firstly, potassium chloride solutions were utilized to determine the frequency that offered the highest sensitivity and the lowest deviation. After establishing this parameter (600 kHz), amplification assays were conducted. In the LAMP assay, using 3.2 fg of DNA template gave the highest fluorescence signal with HNB at the start of the reaction and gradually declined to its lowest level by the end of the reaction. No noticeable differences in HNB color were identified on the paper disc by the naked eye. However, fluorescence changes were obviously noticeable for up to 30 min. The calculated signal strength curve peaked at 60 min, suggesting the reaction continued up to this point[104].

In Spain, PCR is usually used to diagnose congenital Chagas disease, while the applied specific serological tests are among the best performing testing tools, the LAMP assay demonstrated the highest level of analytical performance. The LAMP kit performance was evaluated using visual assessment and a real-time fluorimeter. For congenital Chagas the assay yielded 97% sensitivity while for chronic Chagas it was 47%. The specificity of *T. cruzi* LAMP in congenital Chagas was different among the two different detection methods, while specificity of chronic Chagas LAMP assay was the same by the two different methods. *T. cruzi*-LAMP in congenital Chagas disease showed higher level of agreement with PCR assays than for chronic Chagas disease[105]. Recently, researchers validated a colorimetric LAMP assay for diagnosing *T. cruzi* infection. The assay could identify as low as two DNA copies per reaction across three various *T. cruzi* populations. The selectivity was assessed by testing other protozoa and efficiently identified *T. cruzi* DNA in human blood, achieving detection down to 1.2 parasites per reaction. Additionally, a blinded study was done using canine blood specimens, confirming 100% accuracy, sensitivity, and specificity for the target LAMP assay. Heated 3D printer surface and an insulated container were used to assess the incubation of LAMP and the results demonstrated that it could be applied with affordable materials[106].

Giardia lamblia

A comparative study of 3 molecular techniques for identification of *G. lamblia* DNA in surface water in Poland accomplished the detection of DNA in 15 samples (42%) by LAMP assay. Real-time PCR showed DNA in 12 samples (33%) while nested PCR detected amplification products in 9 samples (25%). These results indicated that LAMP assay was more sensitive than other molecular assays confirming its value in detection of giardiasis[107]. LAMP assays were optimized for detection of *Cryptosporidium* spp. and *G. lamblia*. and the target genes were *SAM-1* gene of *Cryptosporidium* spp. and the *EF1-α* gene of *G. lamblia*. The detection limits were estimated for both specific DNA and low numbers of oocysts and cysts exposed to rounds of freeze-thaw cycles. A multiplex LAMP was applied, capable of detecting both organisms simultaneously. The multiplex LAMP successfully identified DNA from samples seeded with 1 to 4 oocysts and cysts in both clean and surface water[108].

Lalonde and co-authors[109] refined washing procedures for detecting *G. lamblia* cysts from leafy greens and evaluated an EF1α LAMP assay, in comparison to the nested PCR method. The former had higher sensitivity in detection of *G. lamblia* cysts and could also detect cysts on aged and deteriorated specimens. Conversely, nested PCR demonstrated superior sensitivity in samples with fewer inhibitors, and its directly sequenced products are generally more trustworthy for confirmation or additional molecular investigation instead of sequencing LAMP products. However, LAMP assay was considered more convenient offering a shorter reaction time thereby reducing work time in the laboratories. A total of 35 fecal samples proved positive for *G. lamblia* by microscopy, 41 samples were positive by nested PCR targeting the triosephosphate isomerase gene (*tpi*), and 43 samples were positive by real-time LAMP based on the elongation factor 1-alpha (*EF1α*) gene as a target. Both microscopy and nested PCR identified 33 positive samples, while microscopy and real-time LAMP recognized 35 positive samples, and results of both nested PCR and real-time LAMP were the same, detecting 41 positive samples[110].

Trichomonas spp.

Vaginal swabs were tested by culture, PCR, and LAMP techniques. The LAMP showed high sensitivity (42.06%) compared to PCR (7.44%) and culture

(8.26%). No cross-reactivity was detected with *T. tenax* and *P. hominis, S. aureus* and *C. albicans*. The LAMP assay had a low limit of detection (0.036 ng/µl) based on comparison with PCR (0.36 ng/µl)[111]. Vaginal swabs were tested for *T. vaginalis* with LAMP assay using primers designed to target the actin gene. The assay had a low detection threshold of one trichomonad and 1 pg DNA. The LAMP assay showed comparable agreement with PCR in comparison with microscopic examination and multiplex PCR. These results demonstrated that the LAMP procedure was highly specific and sensitive so it can be used to assist in prevention of the disease and its adverse effects[112]. A study assessed PCR, LAMP and the Xpert TV techniques in diagnosis of *T. vaginalis* using 28 microscopy-positive cases and 125 microscopy-negative specimens. All three tests showed 100% sensitivity, with excellent inter-rater consistency among PCR and Xpert TV, and good agreement for LAMP compared to both. The specificities were 100% for PCR and Xpert TV techniques, and 99.2% for LAMP assay. The PPV for the assay was insufficient, requiring further evaluation[113].

Adhesion protein 65 (*AP65*) gene was used as a target in LAMP technique for identification of *T. vaginalis*. The sensitivity of LAMP assay targeting the *AP65* gene was 1000-fold higher than nested PCR based on the actin gene as a target. The LAMP assay targeting *AP65* gene showed high specificity and absence of cross-reactivity with DNA of other parasites[114]. A LAMP assay for diagnosis of *T. tenax* was developed and primers were designed to target the ITS and 5.8S rRNA gene. The assay showed 1000 times higher sensitivity than conventional PCR. It could detect as low as 10 fg DNA and demonstrated high specificity. Also, LAMP technique does not necessitate prior extraction of DNA so it can be used in detection of *T. tenax* in regions with poor infrastructure[115].

Entamoeba histolytica

Stem primers were used in LAMP assay targeting the 18S rRNA gene for diagnosing *E. histolytica*. Results were compared with the performance of the published LAMP and PCR tests. The stem LAMP test had a short reaction time around 11 min, and it could detect as low as 10^{-7} using 10-fold dilution series of parasite DNA, while LAMP had analytical sensitivity of 10^{-5} and PCR sensitivity of 10^{-4}. The stem LAMP assay diagnosed parasite DNA in 36 out of 126 cases, while the conventional LAMP identified 20 out of 126, and 17 cases became positive in PCR technique. The stem LAMP test demonstrated 100% agreement in detection of amplification products, whether using fluorescent dye SYTO 9, SYBR Green I, or stained agarose gel. The findings in this publication highlighted that LAMP assay is sturdy, straightforward, and useful in amebiasis epidemiology detection[116]. The serine-rich protein gene of *E. histolytica* was used to design a set of primers for LAMP assay. The LAMP performance was compared with conventional, nested, and qPCR. The primers showed 100% specificity in LAMP assay while they needed some modification in PCR tests. Three different methods were used to detect amplification products. A single *E. histolytica* trophozoite was established as the lower detection limit for all three post-LAMP detection methods when examined with *E. histolytica* DNA purified from spiked stool specimens. LAMP assay outperformed all PCR tests in the detection threshold[117].

Acanthamoeba spp.

The sensitivity and specificity of colorimetric and fluorescent LAMP techniques for diagnosing *Acanthamoeba* spp. was assessed. *Acanthamoeba* DNA was serially diluted from 1000 pg down to 0.001 pg. Both colorimetric and fluorescent LAMP assays could detect 1, 10, 100 and 1000 pg/reaction of its DNA. The assay could detect as low as 1 pg per reaction and it achieved 100% specificity[118].

Microsporidium spp.

A study focused on developing a LAMP method for the rapid identification of *E. bieneusi*. DNA was purified from thirty samples, all of which tested positive by nested PCR amplification. The conserved region of the small subunit rRNA gene was the target in LAMP assay. The LAMP reaction was optimized and carried out at 63°C for one hour. The diagnostic performance of LAMP assay was evaluated, in comparison to real-time PCR. The LAMP assay had 83.3% positivity and showed no cross-reactivity with other pathogens. Additionally, the assay demonstrated a detection sensitivity of 34 attograms per microliter of total DNA. Real-time PCR successfully identified all 30 specimens verified by nested PCR, whereas the LAMP assay failed to detect all specimens confirmed positive by nested PCR[119].

CONCLUDING REMARKS

1. In malaria, LAMP was used to detect mutations linked to artemisinin and chloroquine resistance. It was proven effective when applied to DBS, making it suitable for resource-limited settings. For *P. falciparum*, a portable LAMP device enabled point-of-care malaria detection. Pipette-assisted isothermal probe amplification achieved malaria diagnosis within 60–80 min. Additionally, MG-LAMP and photo-enhanced PCR techniques showed promising results for malaria detection in military personnel. Besides, LAMP demonstrated an exceptional detection threshold in identifying low parasitemia.
2. It successfully detected *T. gondii* in various samples, including amniotic fluid, air specimens, placental samples, DBS, and brain homogenates. A visual LAMP achieved 100% specificity and 80% sensitivity. The LAMP-LFD procedure enabled detection in primary healthcare settings and remote environments.
3. The stem LFD LAMP assay for *Cryptosporidium* spp. detected fewer oocysts (10/ml) than other molecular techniques. Additionally, a rapid colorimetric LAMP test achieved an analytical sensitivity of one plasmid copy per reaction, surpassing real-time PCR.

4. In leishmaniasis, LAMP retained high detection sensitivity when applied to whole blood, tissue biopsies, and saliva. It effectively identified DNA from various *Leishmania* species, achieving a detection limit of 1 fg for *L. infantum*. A pan-*Leishmania* LAMP test was also introduced for CL and VL diagnosis. A portable LAMP system successfully detected VL and PKDL, with high sensitivity and specificity.
5. For trypanosomiasis, LAMP assay could detect African trypanosomiasis in low-infrastructure regions by utilizing a single-tube dry reagent format with trehalose. A dry-format LAMP assay for *T. evansi* detection was capable of identifying as few as one parasite per assay. Besides, it was validated for *T. cruzi* detection, showing potential as a rapid diagnostic tool.
6. In giardiasis and trichomoniasis, it proved more sensitive for diagnosis than real-time PCR and nested PCR.
7. For *E. histolytica*, the stem LAMP assay exhibited a rapid reaction time around 11 min and had a low detection limit. Three different detection methods confirmed amplification, all capable of detecting a single *E. histolytica* trophozoite.
8. Foe free-living amoeba, LAMP assays surpassed all PCR-based methods in detection threshold, *i.e.*, both colorimetric and fluorescent LAMP assays successfully detected *Acanthamoeba* DNA at concentrations of 1, 10, 100, and 1000 pg per reaction.
9. A LAMP assay exhibited 83.3% positivity with no cross-reactivity in detecting *E. bieneusi*.

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