Chapter 7

Loop-Mediated Isothermal Amplification: An Advanced Method for the Detection of *Giardia*

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Abstract

This chapter provides a reliable and quick method for detection of *Giardia duodenalis* (which causes a dangerous diarrheal disease), prevention of further spreading, identification of the source of contamination, and eventually minimize health risk and economic damage normally caused by an outbreak. The loop-mediated isothermal amplification (LAMP) method is based on the enrichment of parasite-specific nucleotide sequences, similar to PCR, but it is significantly faster and less susceptible to interference. Here, we give an overview of how we developed this method, and using the example of *G. duodenalis* as a water-associated pathogen, we present an optimized examination scheme for its detection in water. For this purpose, we have analyzed data from extensive electronic libraries PubMed®/MEDLINE®, filtered out relevant articles with a keyword search, and summarized them. The number of publications on LAMP method has generally increased steadily since its first report in 2000. LAMP, used for detection of *Giardia*, especially surpasses all other methods due to the high specificity, sensitivity, robustness, and cost effectiveness. The ever-increasing number of publications on application of LAMP is similar to the development of PCR in the 1990s of the last century. Certainly, the method will be further developed in future, but it already offers many advantages over other methods for effective detection of *G. duodenalis* infections and will therefore certainly gain in popularity.

Keywords: loop mediated isothermal amplification, molecular detection, water, feces

1. Introduction

Among diarrheal diseases, Giardiasis induced by the protozoan parasite *Giardia duodenalis* plays a distinct role for a variety of reasons. Infections of humans and animals with the often

overlooked protozoan parasite *Giardia duodenalis* have been reported worldwide. Giardiasis is mainly contracted with the consumption of food and drinking water contaminated with cysts—the environmentally resistant and dormant form of the parasite. *Giardia* cysts are excreted by livestock, wild, and companion animals. These cysts are equipped to survive in extreme and adverse conditions for a long time. The main route of infection is through spreading by water. Giardiasis often occurs as outbreaks, with devastating consequences on human health causing vast economic damage. And most importantly, prophylaxis in the form of drugs or vaccines is impossible. Unfortunately, the inability to involve improved, sensitive, and specific ways for rapid and reliable detection of *Giardia* using microscopic and molecular methods in different matrixes hamper the tracking of this parasite in the environment as well as in patients. For public health, frequent monitoring of water sources using LAMP as the preferred tool is quite effective in both accuracy and costs and can help to detect the parasite source at the earliest possible to avoid outbreaks.

1.1. *Giardia duodenalis:* genetic assemblages and hosts

Giardia is a Diplomonadida, flagellated protozoan with global distribution. The names *G. duodenalis*, *G. lamblia, and G. intestinalis* are the species names interchangeably used in current literature referring to the same organism. Based on genetic, structural, and biochemical data, they are systematically classified; *Giardia* belongs to phylum: Metamonada, subphylum: Trichozoa, superclass: Eopharyngia, class: Trepomonadea, subclass: Diplozoa, order: Giardiida, and family: Giardiidae [1, 2].

Anthony van Leeuwenhoek documented the genus in 1681 for the first time when he microscopically examined his own stool due to his continuous diarrheal sickness. In 1859, Lambl provided a detailed description of the trophozoite and the genus was named Lambl in honor of his work. Until 1879, the cyst stage of the life cycle was completely unknown awaiting Grassi to describe the robust parasitic stage that did not contain flagella (cysts) [3]. In order to give credit to the French zoologist Alfred Giard, Stiles changed the former name (genus and specific epithet) to *Giardia* [4] in 1915.

The *Giardia* genus can be divided into six different species: *G. duodenalis*, *G. agilis*, *G. muris*, *G. psittaci*, *G. ardae*, and *G. microti. G. duodenalis* can furthermore be divided into assemblages and subassemblages [5, 6]. Eight different *G. duodenalis* assemblages have been delineated (A–H) so far, of which assemblages A (subassemblages AI and AII) and B (subassemblages BIII and BIV) are mainly virulent for humans and are often referred to as "zoonotic" assemblages [6, 7]. Narrow host-adapted specificity has been found in assemblages C and D; dogs and canines with assemblage E and domestic livestock (cats) with assemblage F [5, 8]. Assemblage G is associated with rats and mice, whereas assemblage H infects gray seals and gulls [5].

1.2. The *Giardia* life cycle

The *Giardia* life cycle begins with the oral ingestion of a few cysts (ovoid, about 15 × 9 μm × 3 μm), which are resistant under environmental conditions retaining the infectious nature and are transmitted through contaminated water, food, or fecal-oral route (hands or fomites)

[9, 10]. Acknowledging the resilience of these cysts, the parasite is highly virulent; only 1–10 cysts are capable of causing giardiasis [11]. Following an oral ingestion, the low pH of the stomach acid induces excystation (rupture of the cysts) and duplication (asexual replication) of the cell into two binucleated trophozoites. The process of excystation involves the activation of flagella pushing itself out through the cyst wall induced by the proteolytic activity in the duodenum. Simultaneously, the trophozoite undergoes an asexual duplication resulting in every single cyst producing two trophozoites. The trophozoites attach themselves to the duodenal epithelium with their ventral sucking disk and remain within the lumen of the host's proximal small intestine where they are nutritioned by phagocytosis on the dorsal side of the trophozoite. Freely moving or being attached to the mucosa by a ventral sucking disk, they multiply asexually by longitudinal binary fission resulting in manifold reproduction leading to an invasive growth of the trophozoites in the intestine. Trophozoites at the rectum form a robust cyst wall. The trigger for this encystment is still unclear. The process seems to be a result of exposure to and induction by bile salts, fatty acids, and a more alkaline environment. The trophozoite retracts the flagella and division of the nuclei follows before the cysts are excreted with feces.

1.3. Symptoms of the disease

Giardia has a global distribution and is a major contributor to the enormous burden of diarrheal diseases [5, 12, 13]. Giardiasis is a self-limiting disease in immunocompetent individuals with an incubation period of a few days up to 3 weeks. The clinical manifestation is between 1 and 12 days, rarely exceeding 2 weeks [14–16]. Clinically, asymptomatic giardiasis in immunocompetent individual is possible and is frequently associated with excretion of cysts, which however cannot be avoided. Apart from the assemblage, the symptomatic course of infection is confined to be more susceptible in children and elderly/aged people due to their immune incompetence and other host factors [17]. The main symptoms are diarrhea, bloating, weight loss, malabsorption, flatulence, abdominal cramps, nausea, vomiting, fatigue, anorexia, and chills [18–20]. Treatment with drugs is possible within the course of the disease or in chronic conditions. However, preventive vaccination is unavailable [21, 22].

1.4. Transmission routes of giardiasis

The transmission of cysts is possible by the fecal-oral route, through contaminated food or via water-based transmission. *Giardia* cysts are excreted by livestock, wild, and companion animals and are equipped to survive in extreme and adverse conditions for a long time. The likelihood of distribution and consequently the transmission of *Giardia* cysts shed through feces of wild and/or domestic animals that are evident especially after heavy rainfall or river flooding. Such environmental factors favor their transfer to aquifers, local privately farmed gardens, and open-air greenhouses. Waterborne distribution is estimated to be the main source of infection according to various studies of recent years [12, 13, 23]. The food-borne transmission to humans as well as through consumption of packed salads and/or green leaves by infected food-handlers has been reported [24, 25]. Person-to-person contact among schoolchildren attending day care centers and crosscontamination from the

staff to their households are also possible and have a significant epidemiological impact [26–28].

1.5. Epidemiology

The Robert Koch Institute (RKI) in Berlin is the only public health institute in Germany as well as a global health hub publishing weekly reports about illnesses in the German Epidemiological Bulletin. In 2009 and 2010, about 3500–4000 cases of giardiasis were reported [29]. In 2016, the reported *Giardia* cases were 522, whereas during the first 8 weeks of 2017, the reported cases were 415 equaling to about 50 giardiasis incidences per week [30]. In industrial countries, *G. duodenalis* often occurs after the holiday seasons through returning travelers from foreign countries. The post-travel, persistent diarrheal symptoms in patients are most likely related to giardiasis, which is why it is also called "traveler's disease" [31]. Especially, backpackers transmit the parasite to their homes after returning.

In sub-Saharan Africa (SSA), millions of people die of parasitic diseases annually. This includes neglected tropical diseases (NTDs). The geohelminths (soil-transmitted helminths [STHs]) and the intestinal *G. duodenalis* parasite alone infect hundreds of millions of people in SSA [32, 33].

According to the 2016 Statistical Yearbook of United Nation's High Commissioner for Refugees (UNHCR), forcibly displaced people exceeded the number of 65 million worldwide [34]. Until end of January 2016, more than 60,000 registered unaccompanied minor refugees (UMRs) were living in Germany, of which 1248 UMRs between January 2014 and December 2015 underwent an infectious disease screening. Interestingly, 29.2% (364 cases) were infected with more than one intestinal parasite and 7.6% of whom (95 cases) were diagnosed with *G. duodenalis* by immunofluorescence microscopy [35].

2. Database search and inclusion criteria

To ascertain the progress of the LAMP assay since it was developed by Natomi et al. [36] and further evolved by Negamine et al. [37], we conducted a database analysis by keyword search with a special focus on the genus *Giardia*. Two independent reviewers identified the records through PubMed®/MEDLINE®, the database which is considered to be the global literature, the most reliable source of literature search, and a relevant publication retrieval. Two reviewers independently extracted the data and independently assessed the methodological quality. To our best knowledge, this review aimed to assess all literature wherein the LAMP assay was developed and/or applied for detection of *Giardia* in the scientific field. The extraction of relevant literature and appraisal of the finally listed work was carried out up to March 2017. To collect precise information, the comprehensive search entailed the evaluation of published articles including full texts in the English language and those

meeting the inclusion criteria were considered to be appropriate after a critical review. Our search showed one article published in the Turkish language also. We directly contacted the authors who transmitted the electronic text to us. We were able to assess the contents of the article with a translator's help. The terms "loop-mediated isothermal amplification," "loop-mediated isothermal amplification (and) *Giardia*," "LAMP," "LAMP (and) *Giardia*," "polymerase chain reaction," "polymerase chain reaction (and) *Giardia*," "PCR," and "PCR (and) *Giardia*" were entered into the search box, and the articles were processed for further data extraction.

2.1. Results of the literature search

In total, 1850 (0.36%) of extracted articles showed for the term "loop-mediated isothermal amplification" in comparison with 512,447 for "polymerase chain reaction." The LAMP assay was first published in 2000 with a continuous increase in the following years. Until now, LAMP assays have reached the highest level in 2015 with 271 articles published, and thus far until end of March 2017, 110 articles (extrapolated ~440) have been published, which explains the increasing tendency (**Diagram 1**).

Out of 13 LAMP-related articles dealing with the detection of *Giardia*, only six articles were found related to the source/medium: water. Most of the published papers are for the purpose of method evaluation or detection of the targets in patient samples like blood, tissue, and feces. LAMP in water samples was successfully applied for *Giardia* in five articles.

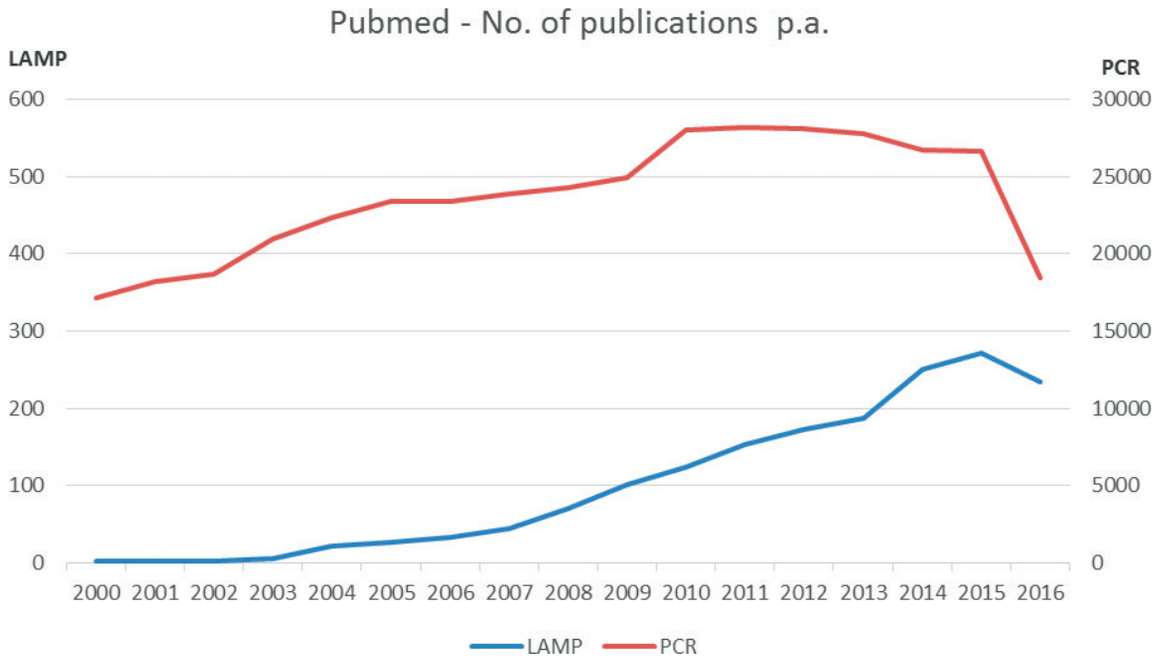

Diagram 1. A comparative graphical representation to illustrate increase in number of publications, LAMP Vs PCR.

3. Loop-mediated isothermal amplification

This review is to introduce the principal concept of a new, advanced, and robust diagnostic method coupled with simplified visualization technique: loop-mediated isothermal amplification (LAMP) with improved sensitivity and specificity for the rapid and reliable detection of *Giardia* DNA.

The LAMP method is a one-step DNA amplification assay performed under isothermal conditions, for 60–120 min using *Bst* polymerase with strand displacement activity and three primer pairs recognizing eight distinct regions within EF-1α (elongation factor-1 alpha) gene for specific detection of *G. duodenalis* (**Figure 1**), producing a considerably high amount of DNA comparable to PCR. The LAMP reaction is carried out in a reaction mixture containing *Bst* polymerase, reaction buffer, primers, DNA template, and a fluorescent dye.

3.1. Primers

LAMP employs two inner primers (FIP and BIP, with typical length of ~40–42 bp), which in turn consists of two parts each and two outer primers (F3, B3 typically length ~ 17–20 bp), which can recognize a total of six distinct regions within the target DNA (see **Figure 1**). The two loop primers employed, forward loop primer (LF) and backward loop primer (LB), were designed to accelerate the amplification reaction and to increase the detection efficiency [37]. In total, six primers recognize eight distinct sites of the target sequence, which can be seen in **Figure 1** indicated as forward (F), backward (B), and complementary (c). In detail, at the 3′ end, the F1c, F2c, and F3c sites are recognized and on the 5′ end, B1, B2, and B3 sites are recognized (**Figure 1**, **Table 1**). The role of F3 and B3 primers is similar to the ordinary and single domain primers used in PCR amplification. They recognize each one of the six regions resulting in amplification of the entire target DNA sequence.

The most common method for designing LAMP primers is the user-friendly online platform: Primer Explorer V4 software (http://primerexplorer.jp/e) running in Java Runtime Environment, a product of Eiken Chemical Co. Ltd. Additionally, Torres et al. developed an extendable LAMP signature design program called LAMP Assay Versatile Analysis (LAVA) necessary for a high-throughput informatics environment, implemented in Perl script with support modules [38]. And lastly, after the completion of the primer design, specificity of the outer primers (F3 and B3) has to be confirmed with a BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in the NCBI database. Several factors are crucial for the performance of LAMP amplification and for optimum primer combinations including GC content, melting temperature (Tm) value, distance between possible primer regions, the stability of primer ends, and ability of possible primers forming secondary structures.

3.2. Mechanism behind the LAMP reaction

The mechanism behind the LAMP reaction involves three major steps: an initial step, a cycling amplification step, and an elongation step.

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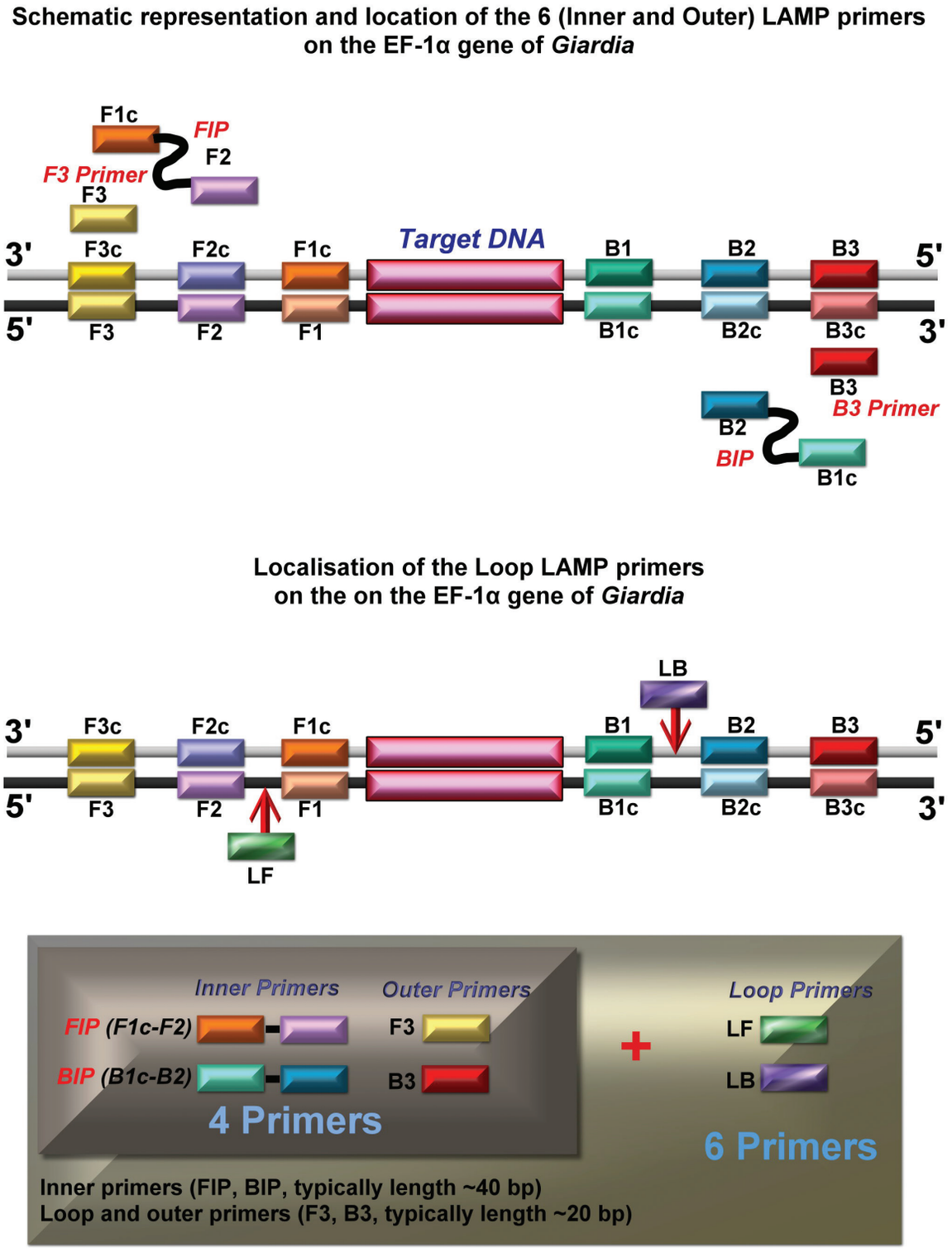

Figure 1. Schematic representation of the three primer pairs recognizing in total eight distinct regions within the EF-1α (elongation factor-1 alpha) gene of *G. duodenalis*.

The simultaneous participation of all six primers is needed for the initial phase production of the starting structure. When the initial phase progresses and during cycling reaction, only the inner primers are used for strand displacement and DNA synthesis. Firstly, one inner FIP

Target	Primer names	Primer sequences	Sequence length	Source/medium	Ref.
Giardia duodenalis EF-1α assemblage B (AF069570)	F3	5′-ATGGACGACGGCCAGG-3′	178 bp	Water, feces, surface water, and sewage samples	[39, 40]
	B3	5′-CCCTCGTACCAGGGCATC-3′			
	FIP	5′-AGCCGATGTTCTTGAGCTGCTT-GTACTCGAAGGAGCGCTACG-3′			
	BIP	5′-GAAGAAGGCCGAGGAGTTCG-TTGTCGGACCTCTCCATGA-3′			
	LB	5′-CTGGACCGGGGACAACA-3′			
	LF	5′-ATCATCTCGCCCTTGATCTCG-3′			
G. duodenalis EF-1α gene	F3	5′- GCCGGGATCTCGAAGGAC-3′	208 bp	Feces pet dogs	[41]
	B3	5′-TCGGGATGTAGTCGAACTCC-3′			
	FIP	5′-T GACCTGGCCGTCGTCCATCTT-GCGACGCTCGCGAACA-3′			
	BIP	5′-G TACTCGAAGGAGCGCTACGAC-GCCTTCTTCCAGCCGATG-3′			
	FLP	5′-GACGGCCAGACGCGCGAG-3′			
	BLP	5′-GCGGAGGGGCTTGTCGGTC-3′			

Table 1. The sequences of the designed primers used for the EF-1α gene of *G. duodenalis* LAMP assays.

(BIP) hybrid primer binds to the starting structure, producing the complementary DNA using *Bst* DNA polymerase. F3 (B3) primer binds immediately after the FIP (BIP) primer, displacing the newly synthesized DNA strand and releasing the target DNA or FIP (BIP)-linked complementary DNA strand. Because of the complementarity of F1c and F1 regions, *Bst* polymerase replaces the F3 site of target DNA sequence with F1c of newly released single strand and forms the initial stem loop-loop structure. Similarly and simultaneously, BIP and B3 primers bind to target DNA resulting in formation of single-stranded dumbbell-like starting structure with loops at both ends. The cycling amplification step uses the single-stranded dumbbell-like starting structure as starting material for further amplification in the LAMP reaction. Only the inner primers (FIP and BIP) are used during the cycling amplification step (**Figure 2**).

3.2.1. Advantages and shortcomings of LAMP assay

The LAMP assay tenders a spectrum of benefits compared to PCR. Even though PCR is sensitive, it has several intrinsic disadvantages, which limit its successful performance. For instance, the presence of inhibitors and other contents like humic acids interferes with environmental samples resulting in a negative impact on the reaction. PCR operates on the principle of denaturation, annealing, and elongation of DNA with a manifold series of repeated temperature changes. This requires an expensive electronically controlled thermal cycler. LAMP, however, runs under isothermal conditions (without complex variable), which only require a water bath or a heat block. Also, failure or not successful performance of the LAMP reaction due to inhibitors is excluded. Last but not least, the turbidity of positive reaction, which could be seen by naked eyes, obviates further visualization steps, e.g., gel electrophoresis (**Table 2**).

LAMP is considered to be field applicable as the read-out of this method is simplified and is based on naked eye visualization: (a) presence of turbidity in sample, (b) colorimetric change in the case of adding metal-ion indicators, (c) presence of fluorescence by adding DNA-intercalating dyes, and (d) confirmation by gel electrophoresis of the final LAMP products that appear as cauliflower-like structures with multiple loops. Recently, Nzelu et al. established a quick, one-step, single-tube LAMP assay combined with Flinders Technology Associates (FTA) card with pre-added malachite green as a direct sampling tool [39].

3.3. Reaction mixture and reaction conditions

Two reaction mixtures have been reported so far for specific detection of *Giardia duodenalis*. The first protocol uses a buffer containing reagents incorporated in the laboratory, whereas the second protocol uses supplied buffer with *Bst* polymerase. It is recommended to use HPLC-purified primers, if not all, at least FIP and BIP as the primers for purity could be crucial for rapidity and reproducibility of amplification.

The LAMP assay developed for first time during 2009 was carried out in a 25 μl reaction mixture containing 1.6 μM each of FIP and BIP, 0.2 μM each of F3 and B3, 0.8 μM each of LF and LB, 2.8 mM of dNTP, 1.6 M of betaine, 20 mM of Tris-HCl (pH 8.8), 10 mM of KCl, 10 mM

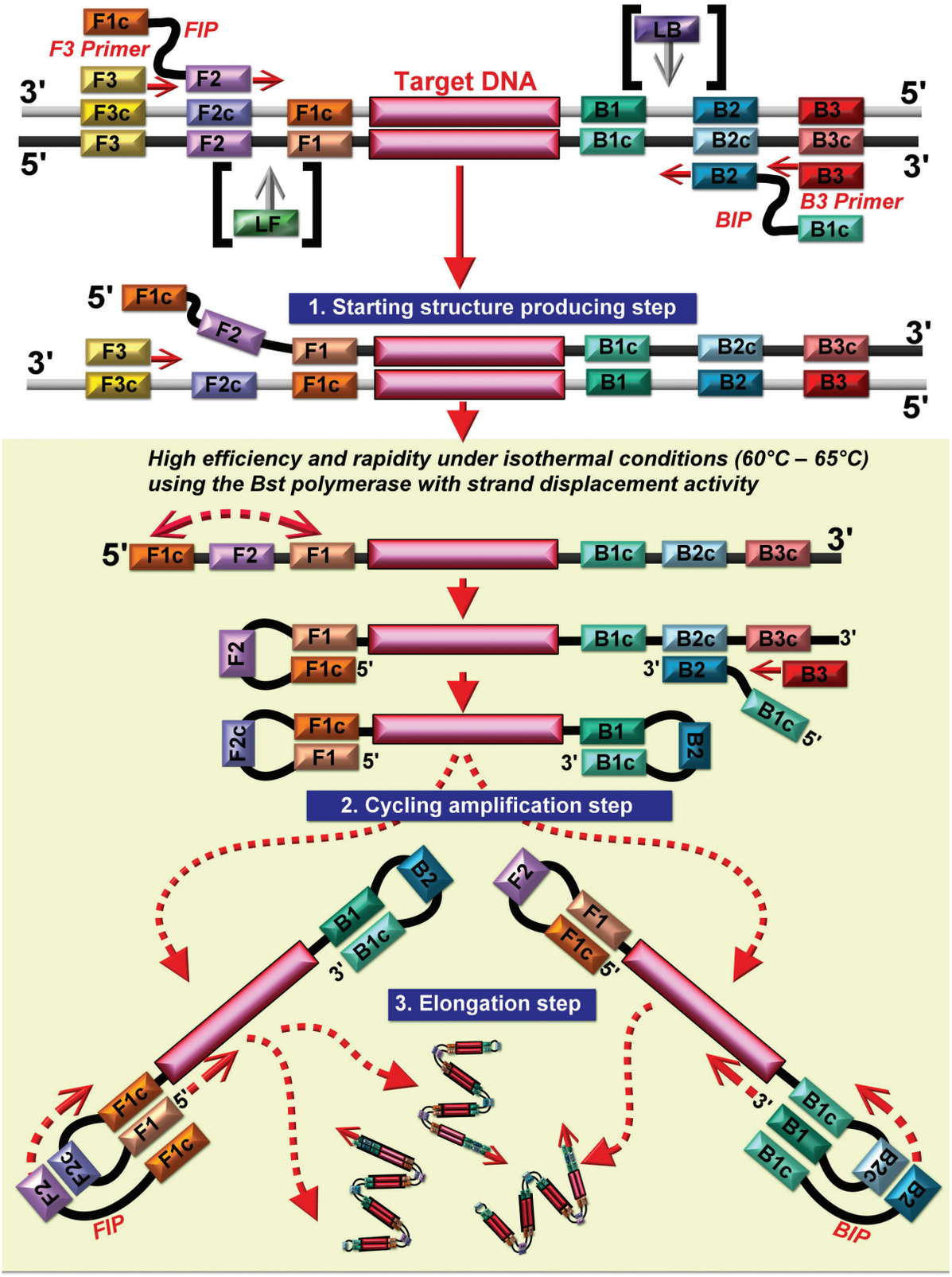

Figure 2. Simplified schematic representation of the major steps in the LAMP method and localization of the eight LAMP primers on target DNA sequence for specific amplification of EF1α gene of *G. duodenalis*.

	PCR	LAMP
Advantages	Sensitive	• Sensitive (10- to100-fold) • Specific (designed to amplify six or eight different regions of the target gene) • Easy • Rapid • Cost effective • Quick
	Genotyping of the amplified product	Isothermal conditions Polymerase with strand displacement activity and no need of heat denaturation of the double-stranded DNA products
	Amplification in thermal cycler, variation in temperature	Amplification in water bath or a heat block Constant temperature Simple and cost-effective equipment
	Interpretation of results in gel electrophoresis	• Interpretation of results by naked eye • Presence or absence of turbidity through production of white precipitate of magnesium pyrophosphate • Colorimetric change after the addition of HNB, malachite green or SYBR green, SYTO-82, SYTO-84, and SYTOX Orange • Fluorescence detection under UV light • Gel electrophoresis • Real-time monitoring turbidimeter • Field applicable
Deficiencies	Only DNA fragments Sequencing of the amplified reaction product	Only DNA fragments Sequencing: possible with limitations
	Time consuming	Multiplex-LAMP difficult
	Inhibitors	Crosscontamination
	Expensive thermal cycler	Need of further progress

Table 2. Advantages and shortcomings of LAMP assay in comparison to PCR.

of $(NH_4)_2SO_4$, 16 mM of $MgSO_4$, 0.2% Tween 20, and the DNA template (2 μl). The reaction mixture was heated at 95°C for 2 min and then chilled on ice. Next, 8 U *Bst* DNA polymerase large fragments were added followed by incubation at 63°C for 120 min and heating at 80°C for 7 min to terminate the reaction [40]. In a consecutive report, the primer concentration was as follows: 40 pmol each of FIP and BIP primers, 20 pmol each of LF and LB primers, and 5 pmol each of F3 and B3 primers [41, 42].

The second protocol was developed in 2013 wherein the LAMP assay was carried out in a 25 μl reaction mixture containing 10× *Bst*-DNA polymerase buffer, 1.6 M betaine, 2.5 mM

each deoxynucleotide triphosphates, 8 mM $MgSO_4$, 0.2 μM each F3 and B3 primers, 1.6 μM each FIP and BIP, 0.8 μM each loop-F and loop-B, 8 U *Bst* DNA polymerase 1 μl of 10,000× concentrated SYBR Green I, and template DNA (2 μl). In this case, the mixture was incubated at 63°C for 60 min and then heated at 80°C for 10 min [43].

3.4. Specificity assessment of the LAMP assay

The specificity of both aforementioned protocols was determined by testing DNA derived from *G. duodenalis* cysts and from phylogenetically related protozoan parasites. This includes *Cryptosporidium parvum, Trypanosoma brucei, Theileria parva, Toxoplasma gondii, Babesia bovis,* plankton biomass, and *G. duodenalis* assemblages A and B for the first protocol [40] and *Toxoplasma gondii, Neospora caninum, Cryptosporidium parvum, Eimeria tenella, and G. duodenalis* for the second protocol [43].

3.5. Sensitivity assessment of the LAMP assay

The sensitivity was assessed using 10-fold dilutions of genomic DNA, and the results demonstrated that LAMP successfully amplified 0.548 pg. DNA/tube (corresponding to ~four cysts) for *G. duodenalis* assemblage B and 0.8 pg. DNA/tube (corresponding to ~six cysts) for *G. duodenalis* assemblage A for the first protocol [40]. The detection limit for the second protocol was 10^{-4} ng/μl (0.1 pg/μl) and 10 times more sensitive than the PCR assay [43].

3.6. Sample collection and purification methods applied in combination with the LAMP

During the development of LAMP methodology for the first time, Plutzer et al. applied it in 10 surface water samples and 15 sewage samples, all collected between 2004 and 2007 in Hungary and previously tested and identified as positive using ImmunoFluorescence Test (IFT) [40, 44]. They also used 10 human fecal samples from Hungarian human patients reported with gastroenteritis in 2007. All samples were amplified by PCRs targeting 18S rRNA [45], glutamate dehydrogenase (GDH) genes [46], triosephosphate isomerase (TPI) gene [47], and EF-1α LAMP. They found that 33 of 35 (94%) environmental and fecal samples were positive for *G. duodenalis* according to one or more of applied techniques. Here, we would like to emphasize that *G. duodenalis*-specific LAMP-amplified DNA was positive in 24 of 35 predefined positive samples, while 23 were positive for 18S rRNA, 15 for GDH, and only 3 for TPI (**Table 3**).

On a more extensive work, the same authors examined 132 aquatic bird fecal samples, collected from February to March 2008 in Hungary [41]. The fecal samples were placed in tubes using polystyrene spatulas and were homogenized in 50 ml of distilled water followed by sieving through 0.1 -mm pore size sieve. After centrifugation, 50 μl of fecal samples were subject to IFT and 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride [DAPI], whereas the remaining part underwent DNA extraction and was subject to 18S rRNA PCR and EF-1α LAMP. Altogether four fecal samples were positive for *Giardia* by IFT, five by PCR, and five by LAMP. Interestingly, *Giardia* in common was identified only in one sample with IFT. In none of the other cases was there a simultaneous/overlap identification of *Giardia* using LAMP or PCR. It is worth to mention that the quality of extracted DNA was assessed in this case with

Matrix/no. of investigated samples	Collection and purification methods	Investigated volume	DNA extraction	PCR target gene	LAMP target gene	Ref.
Surface water (n = 10)	Chemical flocculation or membrane filtration	10–20 L	QIAmp Mini Kit	18S rRNA GDH, TPI	EF-1α[1]	[40]
Sewage water (n = 15)			QIAmp Stool Kit*			
Fecal samples (n = 10)	IMS	10 ml	QIAmp Mini Kit and*			
Fecal samples (n = 132)	Polystyrene spatulas and sieved through 0.1 mm pore size sieve	homogenized in 50 ml distilled water	QIAamp Stool Kit	18 S rRNA	EF-1α[1]	[41]
Drinking water (n = 27)	ARAD microfiber filtration, centrifugation, and vacuum filtration through 3 μm ISOPORE membrane	10–1000 L	QIAmp Mini kit*,**	—	EF-1α[1]	[42]
River water (n = 20)	Membrane filtration (diameter 142 mm), pore size 1.2 μm and sucrose flotation	10 L	QIAamp Mini Kit*	GDH	EF-1α[1]	[48]
Fecal samples (n = 39)	Sieved through four layers of gauze and centrifugation	5 gr	QIAamp DNA Stool Mini Kit	bg	EF-1α[1]	[49]
Fecal samples (n = 72)	flotation technique with saturated zinc sulfate and purification by sucrose gradient	—	QIAamp DNA Stool Mini Kit	Ef-1a (performed with the outer primers B3 and F3)	EF-1α	[43]
WWTPs water (n = 138)	$Al_2(SO_4)_3$ Aluminum sulfate flocculation, and sucrose centrifugation	5 L for influent and 2 L for effluent	QIAamp Mini Kit	16S rRNA	EF-1α[1]	[50]
• Surface water, • Groundwater, raw and drinking water	microfiber filtration (ARAD and Sheather's sugar solution	(a) Up to 400 L (b) Up to 6300 L				
Environmental water samples (n = 420)	$Al_2(SO_4)_3$ flocculation and sucrose flotation	10 L	QIAamp DNA Mini Kit	(SSU)rRNA, GDH	EF-1α[1]	[51]
Drinking water samples (n = 120)						

[1]Primers used according to [40].

*Modification of the manufacturer protocol: addition of ten 10-min freeze-thaw cycles after resuspension in lysis solution.

**Elution with 32-μl LAMP buffer [40 mmol l^{-1} Tris-HCL, 20 mmol l^{-1} KCl, 16 mmol l^{-1} $MgSO_4$, 20 mmol l^{-1} $(NH_4)_2SO_4$, 0.2 v/v % Tween 20, 16 mol l^{-1} betaine, and 28 mmol l^{-1} each deoxynucleoside triphosphate].

-: not reported; bg: beta-giardin; IMS: Immunomagnetic separation (Dynabeads GC-Combo kit, Dynal Biotech); GDH: glutamate dehydrogenase gene; WWTPs: wastewater treatment plants.

Table 3. Results on evaluation studies of the sample collection and purification methods applied in combination with the LAMP in different matrices.

the inclusion of internal controls and identified that their amplification was unsuccessful in 17% of the samples of which nine were positive for *Giardia* by LAMP [41].

To clarify the role of sample inhibitors, 27 drinking water samples of 10–1000 L were collected over a 24-h time period using the ARAD filtration system and were spiked with 100 *G. duodenalis* cysts. The genomic DNA from the samples (water spiked with *G. duodenalis* cysts) was extracted and then EF-1α LAMP was performed. The results showed that LAMP reaction was not affected by inhibitors in any of the samples tested [42].

In total, 10 L Iranian surface water samples from two rivers, collected over a time period of 12 months, were filtered using 142 mm membrane filters and were comparably investigated using IFT, PCR targeting the GDH gene, and LAMP targeting the EF-1α gene. Prior to genomic DNA extraction using the QIAamp Mini Kit, all river water samples were purified through sucrose flotation. The prevalence of *G. duodenalis* cysts was 13 out of 20 water samples by IFT, 10 out of 20 by the GDH gene PCR, and 8 out of 20 by EF-1α gene LAMP assay [48]. Notably in this study, the recovery rate of the protocol was assessed in 5 L water samples, seeded with 5 and 10 cyst/L, and they reported that the mean recovery rate for *Giardia* cysts in the seeded water samples was 18% and all of them tested positive by PCR and LAMP analysis.

During 2015, Çiçek and Şakru used effectively *Giardia* LAMP assay in 39 human fecal samples obtained from Turkey [49]. They primarily screened the patient's fecal material microscopically in native and stained with lugol iodine method to determine the cyst density. After that, samples were subject to DNA extraction using QIAamp DNA Stool Mini Kit and tested for EF-1α gene using LAMP for *Giardia* and beta-giardin (bg) PCR. EF-1α gene LAMP and bg gene region PCR for detection of *G. intestinalis* were found positive in 32 (82%) and 19 (48.7%) of the cases, respectively. Interestingly, the authors stated a significant difference between patients with higher cyst density and lower cyst density ($p = 0.0001$) through the PCR positivity rate [49].

An existing literature documents the performance of EF-1α gene LAMP for detection of *Giardia* in environmental water samples in Germany [50]. The investigators of this study examined a wide palette of different water types and compared the effectiveness of three detection methods: IFT, PCR, and LAMP. A total number of 185 samples originated from influent and effluent wastewater treatment plants (WWTPs), surface waters, a recreational area, groundwater, untreated water from a drinking water plant, and tap water were analyzed during the period from July 2009 to January 2011. For the extraction of the genomic DNA of all sample types, QIAamp Mini Kit was used [50]. All the samples were investigated by three detection assays: IFT, 16S rRNA by PCR, and EF-1α gene by LAMP. The comparison of the three methods indicated better results with IFT compared with the DNA-based assays, among which the LAMP assay was more sensitive than the applied PCR for detection of *Giardia*. The ranking results were as follows: IFA over LAMP and LAMP over nested PCR (56.8 > 42.7 > 33.5%, respectively). Despite nonconcordance of the methods resulting from statistical calculations, the authors outlined differences considering analytical steps such as sample preparation, DNA extraction, and analytical targets. A further explanation closely related to the variable detection capabilities of the assays according to authors is that the samples might contain *G. duodenalis* assemblages other than A and B, which might not be detected by LAMP but may be detected by PCR and/or IFT. The authors in this case speculated

a little further over data interpretation and concluded that another unambiguous factor for the superiority of IFT over the other methods is also possible as IFT detects at the taxonomic level of respective *Giardia* genera and the assemblages cannot be discriminated by this method.

Between 2012 and 2014, Koloren et al. collected 420 environmental and 120 drinking water samples from Turkey [51]. All samples were collected by $Al_2(SO_4)_3$ flocculation and were purified by sucrose flotation technique. DNA isolation was conducted in the purified samples according to QIAamp DNA Mini Kit protocol, and they investigated all samples using EF-1α gene LAMP, small subunit (SSU) rRNA, and GDH PCR. A total of 141 (58.7%), 125 (52.1%), and 120 (50%) were identified positive by each of the aforementioned methods, respectively [51].

Li et al. developed an alternative protocol, including new primer pairs detecting the EF-1α gene of *Giardia*, with potential application for clinical diagnosis of *G. lamblia* from dogs' feces. They collected feces from dogs and processed them by flotation technique with saturated zinc sulfate and purification by sucrose gradient solution. To obtain the genomic DNA template, purified cysts from all fecal samples were subject to QIAamp DNA Stool Mini Kit. The results of microscopy, PCR (performed with the outer primers B3 and F3 of the LAMP assay), and EF-1α gene LAMP for *Giardia* were compared and the results showed that 5 (6.9%) of the 72 dog fecal samples tested positive by microscopy, and 7 (9.7%) and 8 (11.1%) tested positive by PCR and LAMP, respectively [43].

Thoughtfully, we are describing the results of an investigation of Nago et al. (unfortunately, whose contents, preparation steps, and details of the full text are not at our disposal) [52]. They reported that they developed a LAMP assay capable of detecting 3.12×10^{-1} *G. lamblia* cysts per reaction in spiked fecal specimens. Out of the 19 spiked samples, 16 (84%) were successfully amplified by LAMP assay and resulted in positive readings. Furthermore, they attempted to ascertain the negative reaction result in three fecal samples, which is likely due to inhibition. For this, they investigated two specific parameters: dilutions of extracted DNA and addition of bovine serum albumin (BSA) to the LAMP reaction mixture. This modification seemed to yield positive results and to have positive effect on the occurrence of false-negative readings.

3.7. The current momentum toward LAMP

G. duodenalis is one of the most prominent waterborne parasite worldwide and causative agent for several outbreaks in developing, developed, and industrialized countries with fatal consequences, mostly affecting the weakest of the population [12, 13]. The lack of sanitation and health care in Third World nations where malnutrition due to scarcity of food is common leads to highest prevalence of giardiasis in the population. As is often the case, the most vulnerable population groups are also the worst affected: children under the age of 5 years, elderly, and immunocompromised people. Particularly, the mortality rate is correspondingly and shatteringly high among these groups. As a result, scientists and politicians should be encouraged to counteract this dilemma at all levels. Key measures not only include the establishment of appropriate hygiene measures and sanitary facilities and access to clean water but also, or in particular, the setting up of surveillance systems and monitoring programs.

As is often said, prevention is better than cure. However, scanning the objective slides with a microscope is time consuming and exhausting. Cysts could be covered in debris or if at all when available for examination, each cyst will have to be checked for different morphological characteristics, and therefore, skilled technicians are needed. Due to the visualization difficulties of microscopic readings from samples, significant progress has been made in molecular methods such as PCR and PCR-RFLP aiming at proper characterization of *G. duodenalis* into its different assemblages and subassemblages. Therefore, researchers are frequently confronted with the challenge of defining new methods, specific for rapid and accurate diagnosis and for tracking the source of contamination. This is necessary in order to provide efficient treatment and prevent grievance. Even though we have managed to overcome some of the upcoming obstacles, the presence of inhibitors, low sensitivity of molecular methods, and lack of inter- and in some cases intralaboratory standardization in PCR methods are the main reasons that urge scientists to develop further methods.

Water is worth protecting and is the most important nutrient. Contamination of water by *G. duodenalis* is a health risk to all of us. Infective stages of *Giardia* species are able to persist in the aquatic environment for months, which is also the major route of infection. The fast and reliable detection of the parasites and ability to trace its origin can curb the occurrence of larger outbreaks or epidemics premature or better, even avoid one.

With this chapter, we would like to emphasize how effective the innovative LAMP process is. It is worth to be presented to a large specialist audience: one because it offers many advantages over other detection methods and secondly as it is very efficient and easy to carry out without the need for expensive equipment. Moreover, in this case, it is irrelevant that test matrix available for analysis. The detection is easy in stool and tissue samples as well as in environmental samples, mud, and water.

The chapter summarizes all relevant information on the detection of *G. duodenalis* with the LAMP procedure and gives a comprehensive overview of the current state of the art. This is a collection of all available protocols related to the development and application of a simple field-usable method that can meet the needs for a quicker and objective readout for the diagnosis of giardiasis in water and feces. The LAMP assay is ranked among the most accurate molecular tools thanks to its high diagnostic sensitivity and specificity. The future utility of a simple portable device (tube scanner) in which both the amplification platform (heating block) and fluorescent detection unit for end point use (with the ability to acquire real time data) has been envisioned to be combined into a single unit for LAMP assay for the detection of *Giardia* infections.

At present, LAMP is entering the ranks of the recognized detection methods among the World Health Organization (WHO) collaborating centers on foodborne trematode infections. This has been achieved mainly after the reported diagnosis of *Opisthorchis viverrini* infection in stool samples by the use of the LAMP technique [53]. The establishment and the use of a commercial LAMP assay (TB-LAMP) for the detection of tuberculosis was the subject of the expert group meeting organized by the WHO in Geneva in May 2013, and they certified LAMP as a potential diagnostic test. During the last year, CDC-UGA had financially supported the development of RealAmp-LAMP platform for the accurate detection of *Plasmodium vivax* infections [54]. The LAMP is considered as a technology under

development with potential for future application and is currently undergoing large-scale evaluation by the Foundation for Innovative new Diagnostics (FIND) [55] and Centers for Disease Control (CDC) [55]. The proposed method can be expanded to be a quick and specific alternative screening technique for other life-threatening pathogens such as Ebola virus, human papilloma virus (HPV), human immunodeficiency virus (HIV), hepatitis C, etc. Moreover, in case of outbreaks, it could help prevent progression to active disease through early detection in saliva and examine the distribution of pathogens in different body fluids during infectious and noninfectious phases.

The establishment of surveillance activities is the most important step for health care professionals in prevention and in case of outbreaks tracking the source of contamination as fast as possible. Therefore, we advocate for LAMP as a suitable tool, in which given this expense and the large number of ongoing projects addressing, there is clearly a need for the development of a fast, economic assay, and user-friendly approach to detect *G. duodenalis* by fastest possible processing.

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