

Review Article

Loop-mediated isothermal amplification (LAMP) : A rapid molecular diagnosis technique for detection of human pathogens

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Abstract

Delivery of quality healthcare in case of an infectious disease depends on how efficiently and how quickly the responsible pathogens are detected from the samples. Molecular methods can detect the presence of pathogens in a rapid and sensitive manner. Over the years, a number of such assays have been developed. However, these methods, although highly reliable and efficient, require use of expensive equipment, reagents, and trained personnel. Therefore, development of molecular assays that are simple, rapid, cost-effective, yet sensitive, is highly warranted to ensure efficient management or treatment strategies. Loop-mediated isothermal amplification (LAMP), a technique invented in the year 2000, is a novel method that amplifies DNA at isothermal conditions. Since its invention, this technique has been one of the most extensively used molecular diagnostic tools in the field of diagnostics offering rapid, accurate and cost-effective diagnosis of infectious diseases. Using the LAMP principle, many commercial kits have been developed in the last decade for a variety of human pathogens including bacteria, viruses and parasites. Currently LAMP assay is being considered as an effective diagnostic tool for use in developing countries because of its simple working protocol, allowing even an onsite application. The focus of this review is to describe the salient features of this technique the current status of development of LAMP assays with an emphasis on the pathogens of clinical significance.

Introduction

The characterization of pathogens from clinical samples is often time consuming and tedious because it requires multiple steps such as enrichment, growth in selective media, and finally biochemical and/or serological identification. In recent years, due to the advancement in molecular biology, a number of alternative techniques have been developed for rapid and sensitive detection of pathogens⁽¹⁻⁹⁾ However, these methods, although highly reliable and efficient, require use of expensive equipment, reagents, and trained personnel. Therefore, the need of the hour is to employ the tools and techniques of modern molecular biology towards developing assays that are simple, rapid, cost-effective, yet sensitive. Such assays hold tremendous potential as they can support and strengthen the surveillance and monitoring systems of important

pathogens very effectively, particularly in economically backward countries, where the majority of the population are susceptible to the infection by pathogenic organisms due to compromised healthcare systems and lack of general awareness on hygiene and safety.

Among the various molecular techniques, available at present, polymerase chain reaction (PCR)-based detection is considered the gold standard in molecular diagnostics and there are a number of such assays that have commercial applications in routine diagnosis of infectious agents. However, this requires a thermo cycler and a system to detect amplification products. Conventional PCR is being replaced by real-time PCR due to higher sensitivity and ability to detect products in real-time. But this requires expensive equipment. A technique that has shown great

promise in the field of molecular diagnostics is known as loop-mediated isothermal amplification (LAMP), a method of nucleic acids amplification under isothermal conditions⁽¹⁰⁾. The most distinctive advantages of this technology over the widely used conventional technique to amplify DNA (PCR-based techniques) are its simplicity and its rapidity. LAMP relies on an auto-cycling strand displacement DNA synthesis by *Bst* DNA polymerase with a set of four specific primers that recognizes a total of six distinct sequences on the target DNA⁽¹⁰⁾. Isothermal amplification generates many different sizes of stem-loop DNAs containing several inverted repeats of the target sequence. When visualized by agarose gel electrophoresis, these stem-loop DNAs appear as several bands of different sizes in a single well. The amplification of LAMP products can also be determined visually by observing the turbidity generated from white precipitate of magnesium pyrophosphate during the strand displacement, either by naked eye or under UV light when stained with SYBR green 1, or by real time monitoring by Loop amp Turbidi meter.

Principle of LAMP

LAMP, as the name implies, generates multiple copies of a DNA segment at a constant temperature identified by a set of at least four specific primers using a DNA polymerase that possesses the ability of displacing the synthesised strand, which then becomes the template for the next set of amplification^(10, 11). In principle, a forward inner primer containing both sense and antisense sequences of a part of the target DNA region binds and allows the synthesis of the first strand in the LAMP process. Once the first strand is synthesised, the outer forward primer binds and the polymerase displaces the strand. Since the displaced strand contains complementary sequences of the original strand, a stem-looped DNA structure is formed at one end of this strand. This initiates a process in which a backward inner primer hybridizes to the other end of the synthesised strand and results in strand displacement by a backward outer primer to form a dumbbell shape, which means stem-loop structure at both ends of the amplified DNA. This process continues in subsequent LAMP cycles resulting in final products of multiple stem-looped DNAs⁽¹¹⁻

¹⁴⁾. Unlike PCR where only a set of primers is sufficient to amplify a segment of DNA, the LAMP process require four basic primers, which are absolutely critical for loop formation. This ensures high specificity and no amplification is possible in absence of any of the four primers.

Primer design

The performance of LAMP technique depends on a very precise crafting of four specially designed primers. As shown in Figure 1, the primers are F3 (Forward outer), B3 (Backward outer), FIP (Forward inner) and BIP (Backward inner) primers. These primers recognize a total of six distinct nucleotide sequences (B1, B2, B3, F1, F2, and F3) on the target gene. The F3 and B3 play a role in strand displacement and are called as strand displacing primers whereas the inner primers FIP and BIP help in loop formation⁽¹⁵⁾. The FIP and BIP are designed based on six target regions present on the gene: F3c, F2c, and F1c which are in 3' side and B1, B2, B3 in the 5' side of the DNA. In addition to these four specific primers, two more primers called loop primers (LF, Loop forward and LB, Loop backward) can also be incorporated to accelerate the LAMP reaction⁽¹³⁾. Uses of loop primers can reduce the time required to half, and make it a time-saving rapid and efficient diagnostic method. The primers are designed using the Primer explorer software, version 4 (<http://primerexplorer.jp/elamp4.0.0/index.html>).

Basic working protocol of LAMP

LAMP uses *Bst* DNA polymerase, a polymerase that possesses strand displacement activity. Usually, the presence of target DNA is monitored at isothermal conditions between 60°C and 65°C, which is the optimum temperature for *Bst* DNA polymerase. The temperatures at which the LAMP products show better resolution (intensity of the bands) are selected as the optimum temperature. Similarly, the optimization of the reaction length of LAMP assays involves performing LAMP assays between 15 min and 120 min at the optimum temperature with a 15 min difference between each time point. The time point at which maximum detection levels are reached are

considered the optimum reaction time for the assay. Denaturation of the template DNA is carried out for 5 min at 95°C before starting the LAMP reaction for isothermal amplification⁽¹⁰⁾ and this step can also be avoided by directly using the non-denatured templates⁽¹⁴⁾ Incubation is carried out at 58–65°C for 45–60 min, and the reaction is terminated by heating at 80°C for 2–10 min.

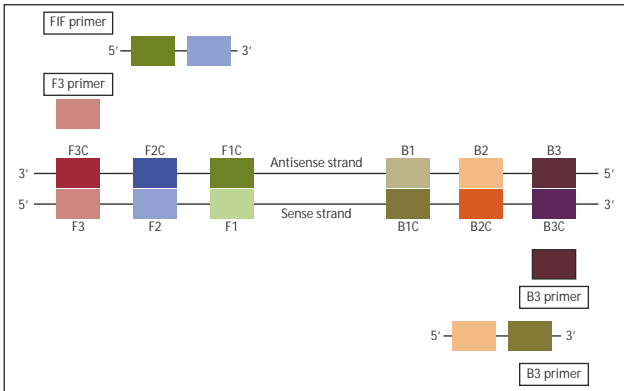


Figure 1 : Schematic representation of the primer design and their positions in the target DNA
 Figure 1 : Schematic representation of the primers in the target gene. The boxes indicates the primers and their positions.

Basic working protocol of LAMP

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End-product visualization

Agarose gel electrophoresis is most commonly used method for end-product visualization wherein the loop DNA's appearing as several bands of different sizes in a single well. Simple visual detection is possible by observing the turbidity derived from the white precipitate of magnesium pyrophosphate generated during the strand displacement by naked eye, or under UV light as bright fluorescence derived from calcein in presence of manganese ions, or in real-time (every 6 sec) by Loop amp Turbidi meter⁽¹⁶⁾.The different methods of end product visualization are shown in Figure 2.

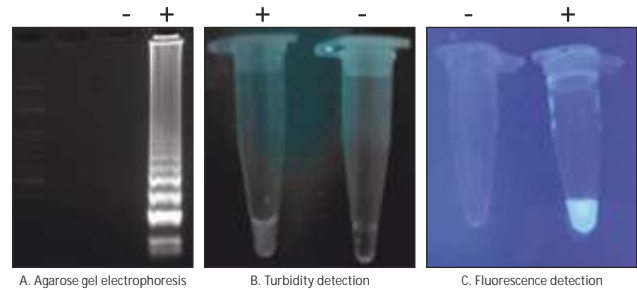


Figure 2 : End product visualization of LAMP technique.

Status of LAMP applications in disease diagnosis

Since the first report in 2000, Loop-mediated isothermal amplification (LAMP) method has attracted a lot of attention around the world, largely due to its potential as a rapid, accurate, and cost-effective molecular diagnostic tool. The technique was first developed in Japan⁽¹⁰⁾ but over the years, researchers in many countries have improvised on the basic protocol and developed various advanced versions of LAMP assays such as reverse transcription LAMP, six primers LAMP, real time Q-LAMP, and Multiplex LAMP. LAMP-based detection methods are now available for a number of bacterial, viral, and parasitic pathogens of humans and many kits have been commercialized⁽¹⁷⁾. More recently, LAMP protocol has been approved as an *in vitro* diagnostic (IVD) tool and adopted as the officially recommended method for routine identification and surveillance in Japan. Currently, LAMP kits for IVD are available for eight pathogens in Japan. These include, Severe Acute Respiratory Syndrome (SARS) coronavirus, *Mycobacterium tuberculosis* (TB), *Mycoplasma pneumonia*, *Legionella* species, influenza type A virus, H1

pdm 2009 influenza virus, H5 influenza virus, and human papilloma virus (HPV) ⁽¹⁸⁾. A comprehensive list of LAMP assays developed for human pathogens are shown in Table 1-3.

Table 1 : LAMP detection assays developed for bacterial pathogens

Bacterial Pathogens	
Species	References
<i>Mycobacterium tuberculosis</i>	Pandey et al, 2008 ¹⁸ , Geojith et al, 2011 ¹⁹
<i>Legionella</i> spp & <i>Legionella pneumophila</i>	Lu X1 et al, 2011 ²⁰
<i>Staphylococcus aureus</i>	Limet et al, 2013 ²¹
Leptospirosis / <i>Leptospira</i>	Sonthayanonet et al, 2011 ²² , Hua-Wei Chen, 2015 ²³ , Koizumi N et al 2005 ²⁴
<i>Listeria monocytogenes</i>	Tanget et al, 2011 ²⁵
<i>Bacillus anthracis</i>	Hatano et al, 2010 ²⁶
<i>Vibrio cholerae</i>	Yamazaki et al, 2008 ²⁷
<i>Clostridium difficile</i>	Boyanton et al, 2012 ²⁸
Candidiasis	Inacio et al 2008 ²⁹
<i>Yersinia enterocolitica</i>	Reza Ranjbar and Davoud Afshar ³⁰
Human/Animal Brucella spp Bacteria	Song L et al, 2012 ³¹
Human/Animal <i>Escherichia coli</i> Bacteria	Hill J et al, 2008 ³²
Human/Animal <i>Trypanosoma</i> spp	Thekiso O Metal, 2007 ³³
Human <i>Giardia duodenalis</i>	Plutzer J et al, 2009 ³⁴
Human <i>Schistosoma mansoni</i>	Fernandez-Soto P et al, 2014 ³⁵
Human <i>Plasmodium falciparum</i>	Najafzadi ZG et al, 2014 ³⁶ , Poon LL Metal, 2006 ³⁷
Human <i>Leishmania</i> spp	Khan M G et al, 2012 ³⁸
Human/Animal <i>Campylobacter</i> spp	Yamazaki W, 2013 ³⁹
<i>Rickettsia</i> spp	Nozomu Hanaoka et al, 2017 ⁴⁰

Status of LAMP in India

During the initial phase of development, LAMP has been applied to many kinds of pathogens causing food-borne diseases ⁽⁶⁰⁾. In India, LAMP assays have been developed mainly for infectious viruses such as chikungunya virus, Japanese encephalitis virus, west Nile, and dengue virus ⁽⁴⁶⁾. We, at Nitte University, are developing LAMP assays for major food borne bacterial and viral pathogens associated with gastroenteritis. We have already developed LAMP assays for detection of *Salmonella typhi* and *Staphylococcus aureus*. We are now working on LAMP based detection of *Shigella* spp. and pathogenic *E. coli*.

Table 2 : LAMP detection assays developed for viral pathogens:

Species	References
H5 influenza virus	Shanthi Jayawardena et al, 2007 ⁴¹
Human papilloma virus (HPV)	Hagiwara M et al, 2007 ⁴²
Chikungunya virus	M. M. Parida et al, 2007 ⁴³
Japanese encephalitis virus	Toriniwa H & Komiya T, 2006 ⁴⁴ , M. M. Parida et al, 2006 ⁴⁵
Dengue virus	M.M Parida et al, 2006 ⁴⁶ ; Teoh BT et al, 2013 ⁴⁷
West Nile	M.M Parida et al, 2004 ⁴⁸ ; Shukla J1 et al, 2012 ⁴⁹
H1N1 Swine flu	Kubo et al, 2010 ⁵⁰
Human Hepatitis B virus	Moslemi E et al 2009 ⁵¹
Influenza A Viruses	Leo L et al, 2005 ⁵²
Human Rubella virus	Abo H, 2014 ⁵³
Human Norovirus Virus (RNA) [69]	Fukuda S et al, 2006 ⁵⁴
Enterovirus	Xia et al, 2011 ⁵⁵
Hepatitis C virus (HCV) genotypes 1-6	Dougbeh-chris Nyan & Kevn L.Swinson 2016 ⁵⁶

Table 3 : LAMP detection assays developed for parasites

Species	References
Cysticercosis (<i>Taenia</i> species)	Nkouawa et al, 2010 ⁵⁷
<i>Strongyloides stercoralis</i>	Pedro Fernández-Soto et al 2016 ⁵⁸
<i>Plasmodium</i> spp	Yee-Ling Lau et al 2016 ⁵⁹

Conclusion

Effective management of any endemics requires three important steps: diagnosis, surveillance/monitoring and prevention/treatment. Diagnosis is the first critical step of disease management. Therefore, development of a sensitive diagnostic tests is very much important and often a limiting step in successful management of infectious diseases. Loop mediated isothermal amplification (LAMP) is a rapid and cost-effective molecular technique that has been utilized for detecting many important pathogens affecting humans. The pace at which the LAMP has been developed for various infectious agents of medical and veterinary importance in the last decade, is a testimony to the potential this technique has and the advantages it holds over other molecular methods. A very precise primer design is a pre-requisite for the use of LAMP. Recent development in the field of molecular biology and biotechnology has made primer designing simpler.

However, the chances of false positivity in a LAMP reaction exist and extensive standardization is necessary before commercializing the developed assays. LAMP is more sensitive and specific when compared to PCR. It has been recommended by both WHO and Office des International Epizootics (OIE) for detection of viral diseases and is an integral part of the pen-side diagnosis. LAMP protocol has also been approved as an IVD tool and has been adopted as the officially recommended method for routine identification and surveillance in Japan. In LAMP, the reactions can be performed and results can be read without the need of expensive equipment. A simple heat block is enough to perform a LAMP reaction. Thus, LAMP has great potential for clinical diagnosis in developing countries, where resources are scarce and the population are exposed to infectious diseases due to non-adherence to strict hygienic practices, especially in rural areas. In this regard, lyophilised LAMP kits which can be stored at room temperature, which have already been developed and are being marketed by commercial entities.

LAMP technique has tremendous application in the field of molecular diagnostics because even a layman, who doesn't possess any prior knowledge about the scientific aspects of DNA amplification, can use this technique. An effective use of LAMP would be in clinical settings, particularly in hospitals and primary health centers, where diagnosis can be done within a few hours of patient walking in. It should also be noted that the developed assays should be cost-effective yet specific, to make them affordable for the common man in a country like India, where the majority of the population is underprivileged and deprived of tertiary health care.

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