



Review Article

Loop Mediated Isothermal Amplification (LAMP) Test– a Novel Nucleic Acid Based Assay for Disease Diagnosis

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ABSTRACT

Disease diagnosis is of crucial importance in making appropriate therapeutic decisions. Conventionally, diagnosis was based on isolation and identification of the causative agent. This was followed in timeline by the development of serological tests. Nowadays, molecular tests credited with high sensitivity like polymerase chain reaction and its variants are replacing the older diagnostic techniques. Major obstacle in adoption of PCR based technique is the need for thermocycler, which precludes its use in low resource setting areas. Isothermal amplification system like Loop mediated isothermal amplification (LAMP) test is a good alternative. It is a novel nucleic acid amplification assay developed in 2000. Amplification of nucleic acid is carried out under isothermal condition, usually at temperature ranging from 60–65°C. Comparatively higher sensitivity, visual detection, lower reaction time, and dispensable use of thermocycler make LAMP an attractive option for field diagnosis. As the test is not susceptible to inhibitory effect of biological substances, it can be very well applied for direct detection from clinical samples without processing. Moreover, quantitation of nucleic acid is possible by real time turbidity measurement. Till date, LAMP tests have been developed for diagnosis of a large number of diseases including bacterial, fungal, viral, and parasitic diseases. Instrument-free systems also have come up. In near future, microchip based LAMP system will be commercially available for point of care testing.

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INTRODUCTION

Diagnosis of disease is important for adoption of proper therapeutic and prophylactic measures. There has been great revolution in the field of disease diagnosis over time. Earlier the basis for diagnosis was isolation and identification of the etiological agent from clinical specimens. Later, serological tests came up as diagnostic tools, which reduced the time for diagnosis. With the evolution of molecular techniques, the time needed for arriving at final diagnosis was cut down to hours from days.

Diagnostic tests based on nucleic acid amplification serve as valuable diagnostic tools, especially in case of diseases where early diagnosis is important factor in determining the final outcome of disease. Moreover, for many of the diseases, isolation of associated etiological agent may be difficult due to fastidious nature which may pose a major hurdle in arriving at proper diagnosis of the underlying cause. In such situations, nucleic acid based detection methods are promising alternative. In 1983, Kary B. Mullis conceptualised Polymerase Chain Reaction (PCR) for the cyclic amplification of nucleic acids, while working as chemist for Cetus Corporation. This was an invention that transformed the field of molecular diagnosis to a great extent and he was awarded with Nobel Prize in Chemistry in 1993. In PCR reactions, two specific oligonucleotide

primers are used to amplify the target sequence. Repetitive cycles of denaturation, annealing and extension are involved in this, resulting in doubling in the number of target sequences at the end of each cycle. There is an exponential increase in the amount of target sequence over time, as it proceeds through different cycles of amplifications (Mullis et al., 1986). Due to advantages such as increased sensitivity, non-culture based amplification, and reduced time for diagnosis, PCR assays were developed for diagnosis of a number of diseases (Wright and Wynford-Thomas, 1990; Lisby, 1993; Rodriguez, 1997; Yang and Rothman, 2004). Also it can be applied for detection of oncogene, antibiotic resistance, epidemiological studies, and genomic fingerprinting (Versalovic and Lupski, 2002; Jannes and DeVos, 2006; Mothershed and Whitney, 2006; Weile and Knabbe, 2009).

Many variants of PCR have been developed for the diagnostic purposes: Multiplex PCR that facilitates simultaneous detection of multiple sequences (Chamberlain et al., 1988), nested PCR with increased sensitivity using two sets of primers, inverse PCR for the amplification of unknown sequences that flank known sequence (Ochman et al., 1988), real time PCR for quantification of nucleic acid (Higuchi et al., 1993). Despite the advantages of PCR based assays like higher sensitivity, the need for costly

thermocycler and post amplification processing preclude its use as routine diagnostic tool in low resource setting laboratories. Rather PCR facilities for accurate diagnosis get restricted to high level specialised diagnostic laboratories.

Several isothermal amplification techniques came up over time: Transcription Mediated Amplification (Guatelli et al., 1990), Strand Displacement amplification (Walker et al., 1992), Rolling Circle Amplification (Fire and Xu, 1995), Helicase Dependent Amplification (Vincent et al., 2004) etc. One of the most widely used isothermal nucleic acid amplification method is Loop mediated isothermal amplification (LAMP). Since its beginning in 2000, LAMP tests have been developed for the detection of a vast variety of disease causing agents. This test has been brought in as an effective alternative diagnostic tool for a number of human, animal as well as plant pathogens. Besides disease diagnosis, this newly developed nucleic acid amplification test has a variety of other applications (Fu et al., 2011). LAMP test can be used for identification of genetically modified organisms – Cauliflower Mosaic Virus 35S promoter gene specific LAMP primers for determining GMO content of Roundup Ready soyabean (Fukuta et al., 2004), GMO specific primers (Lee et al., 2009), GM maize MON863 (Huang et al., 2014); embryonic sex determination (Hirayama et al., 2006; Zoheir and Allam, 2011); meat species identification (Ahmed et al., 2010; Abdulmawjood et al., 2014).

LOOP MEDIATED ISOTHERMAL AMPLIFICATION (LAMP)

Higher sensitivity credited with nucleic acid amplification methods make them attractive options as diagnostic tests.

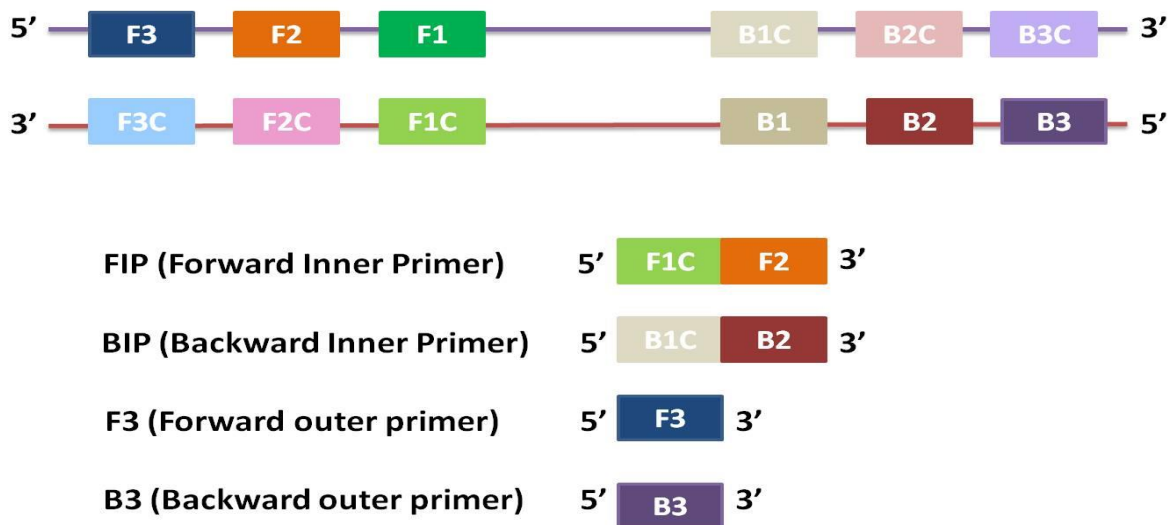


Figure 1: Diagram showing the location of four primers identifying six different regions in target sequence

Major challenge in taking up molecular detection for field level diagnosis is sample preparation for extraction of nucleic acid as PCR is susceptible to inhibitors present in biological samples. The tolerance of LAMP to PCR inhibitors make it suited for direct detection from samples (Kaneko et al., 2007). During the last decade, there have been many reports of development of LAMP tests for disease diagnosis. In the early years after the inception of concept of

But the need for costly equipment like thermocycler restricts their use at field level and laboratories with less facility (Nirju, 2012). Nowadays diagnostic technologies are transforming from benchside to bedside point of care testing (POCT), facilitating easy and early diagnosis (Niemz et al., 2011). Isothermal amplification methods are good options as bedside diagnostic tests. In 2000, Notomi et al. devised the novel isothermal amplification technique LAMP. The basic principle of this technique is autocycling strand displacement DNA synthesis using specific polymerase enzyme with high strand displacement activity like *Bst* polymerase. The major advantage associated with LAMP technique is that nucleic acid amplification can be carried out under isothermal conditions in less time as compared to PCR without compromising the sensitivity and specificity. This makes it adoptable for field level diagnosis. Besides, result can be interpreted based on visual detection which is not possible with PCR.

In LAMP reaction, minimum four sets of primers are used: two outer primers (F3, B3) and two inner primers (FIP, BIP). Designing of primers is of prime importance for the efficient amplification. Target sequence of upto 300 bp is taken and four sets of primers that specifically recognize six distinct regions in the target are designed. Different softwares are available for LAMP primer designing– Primer Explorer (Eiken Co.), LAMP Designer (PREMIER Biosoft International) etc. Incorporation of two more primers i.e. loop primers increase the specificity as well as reduce the reaction time (Nagamine et al., 2002). These loop primers bind to stem-loop structures formed during reaction and further accelerate the reaction.

LAMP, the focus was on the validation of test for identification of different pathogens. But now the interest has shifted to miniaturization of the test in order to make it suited for field level or point of care testing (Mori et al., 2013).

LAMP can be used for amplification of DNA as well as RNA (Laohasinnarong, 2011). Quantitation is possible similar to real time PCR by real time analysis of turbidity

using turbidimeter (Mori et al., 2004). It is cheaper as compared to real time PCR in which fluorescent probes are used. But the turbidity is independent of amplified sequence unlike fluorescent probe. For simultaneous detection of multiple targets, multiplexing can be done (Tanner et al., 2012; Mahony et al., 2013; Yamazaki et al., 2013).

Several LAMP based diagnostic kits are available for disease diagnosis– *Legionella*, *Giardia*, verotoxin producing *Escherichia coli*, *E. coli* O157:H7, *Campylobacter*, *Salmonella*, *Listeria monocytogenes* etc (<http://loopamp.eiken.co.jp/e/products/index.html>). These kits are commercialised by Eiken chemical company, Tokyo. The need for preservation of reagents at lower temperatures is another obstacle in low resource setting areas. Reagents made available in freeze dried form can overcome this (<http://www.freepatentsonline.com/y2008/0182312.html>). Such kits are available for diagnosis of tuberculosis, malaria and African trypanosomiasis, produced by FIND (Foundation for Innovative New Diagnostics) in collaboration with Eiken Company (<http://www.finddiagnostics.org/programs/>).

PRINCIPLE OF LAMP

LAMP reaction mixture consists of DNA polymerase with high strand displacement activity i.e. *Bst* polymerase, four sets of primers– two inner primers and two outer primers, deoxy nucleotide triphosphates dNTPs, magnesium sulphate, betaine, buffer for enzyme, and template DNA. Usually reaction is carried out at 60–65°C for one hour. The target sequence consists of six distinct regions namely F3, F2, F1, B1c, B2c, and B3c in order from 5' to 3' end. Two outer primers are F3 and B3. Inner primers contain the sequence of both sense and antisense strands. FIP contains FIC and F2. Similarly BIP contains B1C and B2 (Figure 1).

Inner primers are added in higher concentration as compared to outer primers. Reaction gets initiated by binding of FIP to complementary sequence in target DNA, which is followed by strand displacement synthesis by outer primer F3. This produces FIP linked complementary strand, with loop at one end. BIP will bind on it, followed by B3. Thus results in the production of a dumb bell shaped structure with loops at both ends. By self primed synthesis, it produces stem loop structure, which acts as starting material for cyclic amplification step. Subsequently FIP binds to it and initiates synthesis by strand displacement producing stem–loop DNA with inverted copy of target sequence at stem and loop involving BIP at other end. On this structure, BIP binds and produces stem loop structure with twice long stem. This process continues. Final product of reaction is a mixture of stem loop DNAs of varying lengths and cauliflower like structures with multiple loops (Notomi et al., 2000). Loop primers will bind to loop regions that are not bound by inner primers (Nagamine et al., 2002).

DETECTION OF LAMP PRODUCT

The foremost advantage of LAMP in comparison with PCR is that the result of amplification can be interpreted without post amplification processing. Visual turbidity is a good indicator of positive reaction. Nucleic acids are amplified in large amount in LAMP reaction. This results in production of large excess of pyrophosphate ions, which will combine with magnesium ions resulting in production of white

precipitate of magnesium pyrophosphate. This is responsible for turbidity in case of positive reaction (Mori et al., 2001). Increase in turbidity will be in direct proportion to the amount of nucleic acid, which can be measured real time using real time turbidimeter. Turbidity is measured as OD at 400nm in every 6 seconds. It is cheaper than real time PCR machine. For the formation of white precipitate, yield in microgram quantities is required. In LAMP, DNA is amplified to more than 10µg, therefore visual turbidity can be used as an indicator of positive reaction (Parida et al., 2008).

Polyethylene imine (PEI) can be added to reaction tube post amplification for the detection of amplification. PEI forms insoluble complex with high molecular weight amplification product, but will not combine with low molecular weight oligonucleotides. Visually detectable clear coloured precipitate is formed on addition of PEI. But PEI cannot be added prior to reaction, as it will inhibit amplification (Mori et al., 2006).

Amplified products can also be visualised in presence of fluorescent intercalating dyes such as SYBR Green I, Calcein etc. On addition of SYBR Green I to the reaction tube post amplification, the colour changes from orange to green in case of positive reaction. Fluorescence can be detected visually using handheld UV torch (wavelength 365nm). Calcein is a fluorescent metal ion indicator. It can be added to tubes prior to reaction. Calcein quenches the manganous ions. Before reaction, solution appears orange in colour. As the reaction proceeds, manganous ions are released from calcein and it will combine with pyrophosphate ions, thus increasing fluorescence of calcein. Increased fluorescence can be detected visually as well as by ultraviolet light (Tomita et al., 2008).

Colorimetric detection is also possible by addition of 120µM Hydroxynaphthol blue (HNB) to the reaction mix before amplification. HNB is a metal ion indicator. The colour of HNB changes depending on pH of the solution. Positive reaction is indicated by change in colour from violet to sky blue (Goto et al., 2009).

Analysis of LAMP reaction products can be done by agarose gel electrophoresis on 2% agarose gel, followed by staining with ethidium bromide solution and visualisation under UV transilluminator. In positive cases, it will produce ladder like pattern due to the production of stem–loop structures with different stem lengths (Parida et al., 2008). Either restriction enzyme digestion or sequencing can be done for the confirmation of specificity of the amplified product in LAMP reaction.

APPLICATIONS OF LAMP AS A DIAGNOSTIC TEST

The shorter reaction time without compromising the sensitivity and specificity, and the independence from the use of thermocycler makes the test suitable for diagnosis in low facility laboratory settings, which cannot afford the high cost equipments. LAMP tests have been designed for the diagnosis of a vast array of diseases (Table 1).

INSTRUMENT FREE LAMP SYSTEMS AS POCT

According to WHO, point of care diagnostic test must be ASSURED (Affordable, Sensitive, Specific, Rapid and robust, Equipment free, and Deliverable to the end user). Isothermal amplification techniques can be adopted for field

diagnosis in equipment free systems, since there is no need for thermocycling. Instrument free, microLAMP systems are

Organism	Author	Gene target
Bacterial diseases		
<i>Mycobacterium</i>	Iwamoto et al., 2003	<i>gyrB</i>
<i>Bacillus anthracis</i>	Qiao et al., 2007	<i>Ba813, pag, capB</i>
<i>Campylobacter jejuni, Campylobacter coli</i>	Yamazaki et al., 2008	<i>cj0414, CCO0367</i>
<i>Yersinia ruckeri</i>	Saleh et al., 2008	<i>yrul/yrur</i>
<i>Escherichia coli</i>	Hill et al., 2008	<i>malB</i>
<i>Pasteurella multocida</i>	Sun et al., 2010	<i>kmt1</i>
<i>Brucella</i>	Lin et al., 2011	<i>omp25</i>
<i>Listeria monocytogenes</i>	Wang et al., 2011	<i>iap</i>
<i>Mycoplasma bovis</i>	Bai et al., 2011	<i>uvrC</i>
<i>Actinobacillus pleuropneumoniae</i>	Ji et al., 2012	<i>dsbE-like gene</i>
<i>Chlamydia psittaci</i>	Lin et al., 2012	<i>MOMP</i>
<i>Mycoplasma wenyonii</i>	Song et al., 2012	<i>16S rRNA</i>
<i>Mycoplasma hyopneumoniae</i>	Li et al., 2013	<i>mhp165</i>
MRSA	Su et al., 2014	<i>orfX</i>
Fungal diseases		
<i>Paracoccidioides brasiliensis</i>	Endo et al., 2004	<i>gp43</i>
<i>Candida</i>	Inacio et al., 2008	<i>26S rDNA</i>
<i>Histoplasma capsulatum</i>	Scheel et al., 2014	<i>Hcp100</i>
Viral diseases		
West Nile virus	Parida et al., 2004	<i>E gene</i>
Norovirus	Fukuda et al., 2006	<i>RdRp</i>
Chikunguniya	Parida et al., 2007	<i>E1</i>
Rift Valley fever	LeRoux et al., 2008	<i>RNA polymerase</i>
PRRS	Gao et al., 2012	<i>M</i>
Porcine cytomegalovirus	Yang et al., 2012	<i>DPOL</i>
Capripox	Das et al., 2012	<i>VP39</i>
Marek's disease	Angamuthu et al., 2012	<i>ME9</i>
Camel pox	Venkatesan et al., 2012	<i>C18L</i>
Mink enteritis	Wang et al., 2013	<i>VP2</i>
Porcine kobuvirus	Li et al., 2014	<i>3D</i>
Parasitic diseases		
<i>Trypanosoma</i>	Thekiso et al., 2005	<i>A1</i>
<i>Plasmodium</i>	Han et al., 2007	<i>18S rRNA</i>
<i>Babesia</i>	Alhassan et al., 2007	<i>EMA-1, Bc48</i>
<i>Cryptosporidium</i>	Karanis et al., 2007	<i>gp60</i>
<i>Toxoplasma gondii</i>	Zhang et al., 2009	<i>529bp sequence</i>
<i>Schistosoma japonicum</i>	Xu et al., 2010	<i>SjR2</i>
<i>Paragonimus westermani</i>	Chen et al., 2011	<i>ITS-2 of rDNA</i>

Table 1: Examples of diseases detected by LAMP assay

well suited for diagnosis in low resource setting areas. Non-Instrumented Nucleic acid Amplification (NINA) for malaria was developed by LaBarre et al. in 2011. They devised NINA heater for carrying out LAMP reaction on the basis of heat generation from exothermic reaction between calcium oxide and water. In order to maintain the temperature, reaction chamber was surrounded with engineered fat substance with melting range at 65°C. Temperature can be maintained for 45 minutes with reaction between 20g calcium oxide and 6.8ml water, which is enough for one LAMP reaction. Result can be interpreted based on turbidity or fluorescent dyes.

The major hurdle in adoption of nucleic acid amplification assays as field level diagnostics is the requirement of nucleic acid extraction. LAMP is well suited for direct detection from clinical samples (Kaneko et al., 2007). There have been reports of direct detection of

Plasmodium falciparum from blood samples by just heating at 99°C for 10 minutes prior to LAMP reaction (Poon et al., 2006). For diagnosis of tuberculosis in developing countries, LAMP for direct detection from sputum samples has been developed by FIND (Foundation for Innovative New Diagnostics) in collaboration with Eiken Co (Boehme et al., 2007). In this, sputum samples are subjected to pretreatment for 7-8 minutes to remove the inhibitory substances and then they are directly added to freeze dried LAMP reagents (PURE-TB LAMP).

ADVANTAGES AND DISADVANTAGES

Following are the points in credit for loop mediated isothermal amplification test as diagnostic test over PCR. Dispensable use of thermocycler makes the test less expensive. Result is obtained in less than 1 hour. Sensitivity is higher than that of conventional PCR. Non-denatured

template can be used for amplification (Nagamine et al., 2001). Amplification is produced under isothermal conditions and can be performed either in less equipped laboratories or at field level, in a water bath. Amplicon can be detected visually using hydroxy naphthol blue, SYBR Green I etc. LAMP is credited with tolerance to inhibitory substances such as culture medium and biological substances which can affect the efficiency of PCR (Kaneko et al., 2007).

In spite of these advantages, there are some drawbacks associated with this technique. Most important problem is the chance of carry over contamination, since nucleic acid is amplified to microgram quantities. By avoiding post amplification opening of the tube, contamination chances can be lessened to some extent. For this, dyes like HNB, calcein etc. can be added to the reaction mixture prior to amplification, as these dyes will not interfere with amplification process. Another strategy is UTP/UNG system used for avoiding PCR carry over contamination. Preincubation with enzyme UNG (Uracil N- Glycosylase) which will degrade the uracil and use of UTP instead of TTP in deoxyribonucleotide mix in reaction prevents contamination from previously amplified products (Dhama et al., 2014). Sample preparation, amplification reactions, and post amplification processing (if needed) must be carried out in different rooms to avoid chances of contamination at all stages. Another disadvantage is that LAMP cannot be used for amplification of sequences of size more than 300bp.

CONCLUSION

In present time, more focus is given to molecular level detection for disease diagnosis. A variety of molecular diagnostic tests have come up in the last few decades. Loop mediated isothermal amplification (LAMP) assay is one of the most important innovative tests developed in the last decade. Since it is carried out under isothermal conditions and result can be interpreted visually, it is well suited for adoption as a field level diagnostic in low resource setting areas in developing countries. Reaction components can be made available in freeze dried form, which will preclude the need for low temperature storage. Various modifications of LAMP like equipment free systems (NINA) have been made for making it suited for point of care testing. In coming time, microchip based LAMP test kits combining nucleic acid extraction, LAMP amplification and detection will be coming up for major animal and human diseases. This will pave a new opening for field level diagnosis in less time with high sensitivity and specificity.

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