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Mayank Kumar Agarwal
Assistant Professor, Department of Veterinary Physiology & Biochemistry, Arawali Veterinary College, Sikar, Rajasthan, India

Narendra Singh Rathore
Assistant Professor, Department of Veterinary Biochemistry, College of Veterinary & Animal Science, RAJUVAS, Bikaner, Rajasthan, India

Amit Kumar Pandey
Assistant Professor, Department of Veterinary Biochemistry, College of Veterinary & Animal Science, RAJUVAS, Bikaner, Rajasthan, India

Corresponding Author:
Mayank Kumar Agarwal
Assistant Professor, Department of Veterinary Physiology & Biochemistry, Arawali Veterinary College, Sikar, Rajasthan, India

Loop mediated isothermal amplification (LAMP) of DNA: An advanced gene amplification technique

Mayank Kumar Agarwal, Narendra Singh Rathore and Amit Kumar Pandey

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Abstract
The amplification of DNA provides method for gene based specific detection. Besides Polymerase Chain Reaction (PCR), amplification can be achieved by several isothermal techniques. The PCR suffers from various drawbacks like requirement of laboratory and expertise to perform. Loop Mediated Isothermal Amplification (LAMP) of DNA is one of the promising isothermal techniques rapidly gaining popularity among researchers. The technique requires Bst DNA polymerase and a set of four primers recognizing 6-8 regions of target DNA. LAMP technique of DNA gained surprising popularity for amplifying a desired gene at constant temperature between 60-65 °C & maintain a constant temperature can be easily fulfilled by a water bath or a heat block. The LAMP amplification of the desired DNA fragment can be achieved as high as 10^9 copies in merely 30 min with higher specificity as compared with the existing PCR technique. The technique of LAMP averts the use of costly thermal cycler instrument, facilitating establishment of a field-friendly technique and is promising technique for gene detection in a resource-limited laboratory.

Keywords: Loop mediated isothermal amplification (LAMP), polymerase chain reaction (PCR), Bst DNA polymerase, forward inner primers (FIP), backward inner primers (BIP), point of care testing kits (POCT)

Introduction
LAMP is a powerful technique for the detection of pathogenic organism or gene thereof in the sample. It overcomes the drawbacks of existing detection techniques *viz*. PCR, ELISA HPLC which require costly instrumentation, pre-treatment of samples, expertise to perform, and an additional course of action to visualize results. The amplification of the desired fragment of DNA can be achieved in a lesser time with higher specificity in isothermal conditions using Bst DNA polymerase, as compared with the existing *Taq* DNA polymerase dependent PCR technique.

The Polymerase Chain Reaction (PCR) requires *Taq* DNA polymerase enzyme for DNA amplification isolated from *Thermus aquaticus* bacteria. The optimum temperature of this enzyme is 72 ℃. So a cycle of three different temperature is required for amplification. The separation of DNA duplex template occurs at ~95 ℃ and annealing of primers to DNA template requires 52-56 ℃ and extension is obtained at 72 ℃. The discovery of Bst DNA polymerase enzyme it has been possible to amplify DNA at constant temperatures between 60-65 ℃. Different isothermal (Karami *et al*., 2011) [5] techniques have been devised *viz*. transcription mediated amplification (TMA) or self-sustained sequence replication (3SR), nucleic acid sequence-based amplification (NASBA), signal-mediated amplification of RNA technology (SMART), strand displacement amplification (SDA), rolling circle amplification (RCA), loop-mediated isothermal amplification of DNA (LAMP), isothermal multiple displacement amplification (IMDA) and circular helicase-dependent amplification (cHDA).

Out of these isothermal techniques, Loop Mediated Isothermal Amplification of DNA (Notomi *et al*., 2000) [9] gained surprising popularity for amplifying the desired gene at a constant temperature between 60-65 ℃, achieved by Bst DNA polymerase-based strand displacement activity. The requirement of this technique to maintain a constant temperature can be easily fulfilled by a water bath or a heat block. Presently, this technique is in the establishment phase and rapidly replacing PCR for amplifying/detecting DNA. LAMP is known as a rapid, robust technique for the detection of any gene or allele in an organism from unprocessed blood/urine biological samples. Dry-LAMP is a modification of LAMP which uses a reaction mixture in dried form and has great potential in developing point of care testing kits (POCT).

Through LAMP, amplification of the desired DNA fragment as high as (10^9) copies in merely 30 min can be achieved with higher specificity as compared with the existing PCR technique. The technique of LAMP averts the use of costly thermal cycler instrument, can be established as a field-friendly technique and is a promising technique for gene detection in a resource-limited laboratory.

Principle of LAMP Reaction

The LAMP reaction requires primers that recognize six distinct regions designated as F3, F2, F1, B1, B2, and B3combined into four primers in a set namely F3, FIP (F1c-F2), BIP (B1c-B2) and B3. Out of these, F3 and B3 are said to be outer primers and required in lesser amounts. (Figure 1) While FIP and BIP are forward or backward inner primers and are the main primers required in higher amounts. The LAMP reaction marks with an initial non-cyclical phase, including DNA synthesis by annealing the F2 region of FIP and elongation through polymerase action, followed by annealing of BIP in a similar manner. The F3 and B3 primer anneals and elongates DNA along with strand displacement to generate single stranded DNA with complementary regions, which leads to the formation of a dumb-bell like structure at both ends. Later in the cyclical phase, the dumbbell shaped, self primed, single stranded DNA undergoes several steps of elongation, eventually generating cauliflower shaped concatamers. (M Parida *et al.*, 2008) [8]

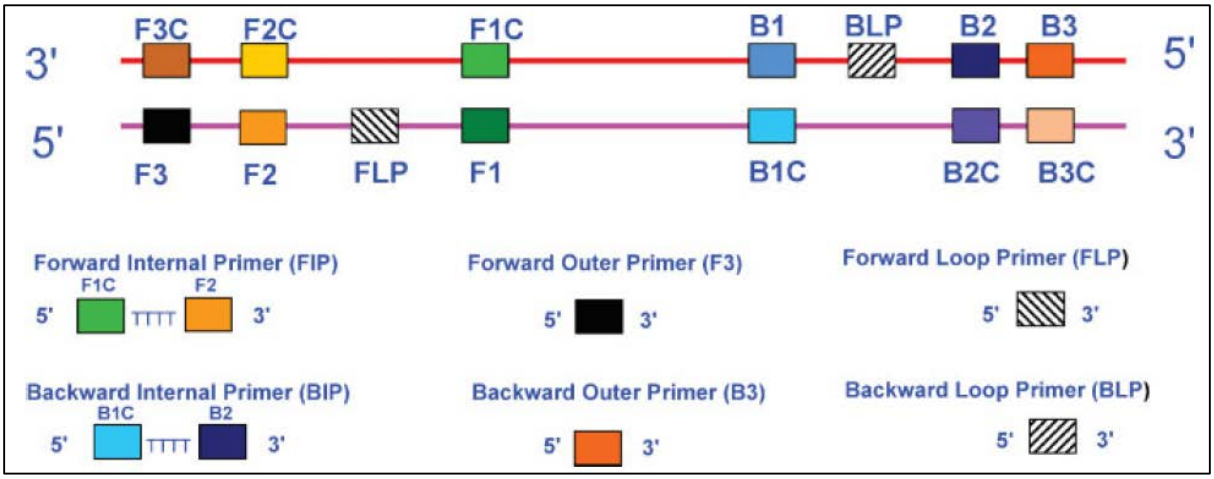

Fig 1: Schematic representation of primer design for LAMP assay showing the position of the six primers spanning the target gene

Materials and Methods

Materials

a. Whole Blood Genomic DNA Purification Mini Kit
b. MgSO4, 100 mM
c. dNTP Mix, 10 mM
d. Primers, HPLC grade
e. Bst DNA Polymerase, 8000 U/ml (M0275L, New England Biolabs)
f. Nuclease free water (NFW)
g. DNA ladders
h. 6X DNA Loading Dye
i. 5 M Betaine (SLBW8334, Sigma)

Equipments

a. Autoclave
b. Water Bath
c. Vortex Shaker
d. Centrifuge
e. Incubator
f. Weighing Machine
g. Gel Documentation System
h. Electrophoresis Unit
i. Thermo Cycler
j. Microwave oven

Methods

Sample collection and DNA isolation

The LAMP technique provides flexibility to use variety of samples in the assay viz. Biological material (blood, urine, CSF, tissue) etc. The samples are transported in ice pack to laboratory and processed for genomic DNA isolation. The isolated DNA is checked for output and concentration through 1.5% gel electrophoresis and Nanodrop.

Primer designing

The nucleotide sequence of the desired gene can be downloaded from NCBI. LAMP primers are designed using PrimerExplorerV5 online software (https://primerexplorer.jp/e/) with default settings and notepad FASTA format of the desired gene as input sequence.

Optimization of LAMP reaction

To optimize the reaction, F3 and B3 primers from different sets of the designed LAMP primers are used as forward and reverse primers for gene amplification in PCR reactions. The isolated genomic DNA is taken as a template. The reaction mixture consists of 3 µL of the genomic DNA template, 0.4 µM of each primer, and 12.5 µL of PCR master mix to make a total of 25 µL. The mixture is subjected to amplification with conditions as shown below:

Table 1: The mixture is subjected to amplification with conditions as shown below

Condition	Temperature	Duration	No. of Cycles
Initial denaturation	95 °C	5 min	1
Denaturation	95 °C	1 min	
Annealing	49-58 °C	1 min	35 cycles
Extension	72 °C	1 min	
Final Extension	72 °C	10 min	

The set for which F3 and B3 primers are working is selected for LAMP reaction optimization. After confirmation of the

primer set, Loop Mediated Isothermal Amplification (LAMP) is optimized with gene specific LAMP primers (F3, FIP, BIP and B3) at several isothermal temperatures (57-67 ℃) maintained by thermal cycler in 0.5 ml PCR tubes. Further, the LAMP reaction is optimized for incubation period of the reaction (20-60 min), MgSO4 concentration (6-10 mM), template concentration (5-50 ng/µl). The LAMP reaction is optimized for primer ratio of FIP/BIP to F3/B3 primers. The ratio of 4:1 and 8:1 is commonly used to obtain better results. (Betaine) (0.5-2 M) can be utilized to increase difference between 'Time to Result' of negative and test sample. The LAMP reaction mixture is as follows:

Table 2: Reaction Mixture for LAMP Amplification-

Composition of LAMP Reaction	25 µl Reaction	Final Reaction
Template volume	1.0 µl	10-40 ng
FIP / BIP (16 µM)	2.5 µl	1.6 µM
F3 / B3 (6 µM)	0.85 µl	0.2 µM
dNTPs (10 mM)	3.5 µl	1.4 mM
Bst DNA Polymerase	1 µl	8 U
10 X Thermopol buffer (µL)	2.5 µl	1 X
$MgSO_4$ (100 mM)	1.5 µl	6mM
Betaine (5 M)	0.5 µl	1-2 M

The results of LAMP amplification is judged through naked eyes without dye or by addition of dyes *viz.* calcein dye, Hydroxy Napthol Blue (HNB), SYBR green or by running the LAMP products in 2% agarose gel electrophoresis.

Interpretation of Results

LAMP products can be visualized in gel electrophoresis assay similar to PCR products. Unlike PCR, the products appear as bands of varying size (Figure 2). However, to avert the post-amplification procedures several methods have been explained. The LAMP reaction concludes with generation of DNA as high as 20 µg/25µL reaction as compared to that of PCR with 0.2 µg/ 25 µL. Such huge amount of DNA can be interpreted through naked eyes as hazy precipitate or using a turbidometer adjusted at 650 nm wavelengths (Figure 3).

To increase the sensitivity of assay, several metal ion indicators can be added before or after the reaction for visualization of LAMP products. The fluorescent metal indicators like calcein, HNB initially remains quenched with Magnesium (Mg+2) ions. The LAMP reaction proceeds through production of pyrophosphate ions as by-product from the reaction substrate, deoxyribonucleotide triphosphate.

These pyrophosphate ions react (Figure 4) with Mg+2 ions to form Mg2P2O7 thereby making indicator deficient of Mg+2, which results in emission of fluorescence. Real time LAMP assay (Figure 5) using RT-PCR machine has proved its utility in determining 'Time to Result' and thereby optimization of the LAMP assay.

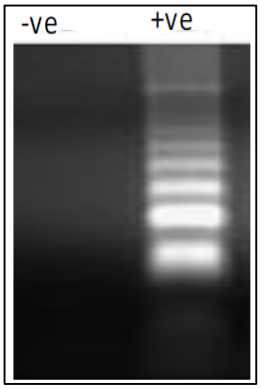

Fig 2: Visualization of LAMP amplified product in gel electrophoresis using ethidium bromide staining showing negative and positive reaction

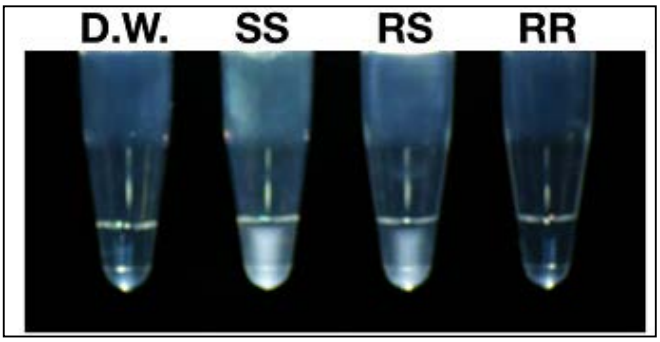

Fig 3: Ocular visualization of LAMP amplified products showing turbidity in positive, while clear reaction mixture in negative reaction

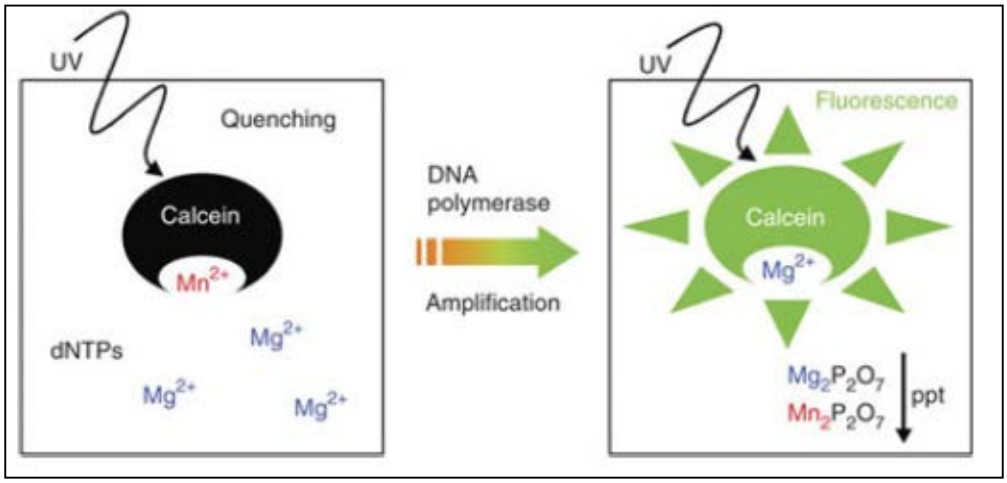

Fig 4: Mechanism showing generation of fluorescence through metal indicators for visualization of DNA in LAMP reaction

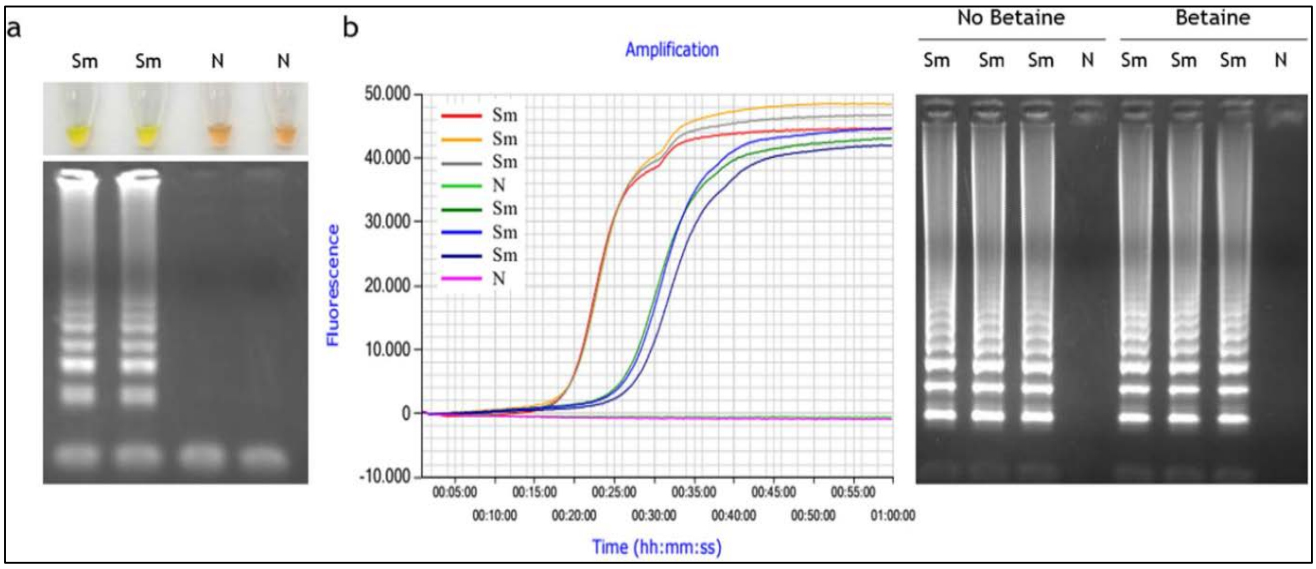

Fig 5: Conventional and real time visualization of DNA amplification in RT-LAMP (Real Time LAMP) assay (Diego *et al.*, 2019) [4].

Application of LAMP Assay

This isothermal amplification method averting sample preparation and post amplification procedures find utility in the area of gene detection or disease diagnosis in all class of organisms. The LAMP assay has been utilized for detection of food and meat adulterations (Liu *et al*., 2017) [6], protozoan infections (Abbasi *et al*., 2016) [1], Mycoplasma infections (Sharma *et al*., 2019) [10], cancer (Wang *et al*., 2020) [12]. Chronic myeloproliferative neoplasms (Minnucci *et al*., 2012) [7] detection of insecticide resistance genes (Badolo *et al*., 2012) [2], sex identification in birds (Chan *et al*., 2012) [3]. Further, the master mix can be mixed with 0.2 M trehalose and desiccated thereafter, for utility of LAMP reaction at point-of-care diagnosis (Thapa *et al*., 2019) [11].

Summary

Loop Mediated Isothermal Amplification of DNA (LAMP) is one of such biosensors recently described by Notomi *et al*. (2000) [9] provides facility of gene amplification/detection and has potential of developing into a point of care testing kits (POCT). It requires a source of constant heating at a defined temperature, Bst DNA polymerase and a set of four LAMP primers capable of amplifying a desired gene within 20 min. LAMP has been utilized for detection of variety of genes and pathogens. Interestingly, LAMP reaction has been standardized for differentiating alleles having SNP in gene sequence which are popularly known as allele specific LAMP. Dry-LAMP is a modification of LAMP with reaction mixture in dried form. The technique of LAMP averts the use of costly thermal cycler instrument, facilitating establishment of a field-friendly technique and is a promising technique for gene detection in a resource-limited laboratory. Hence, LAMP offers a rapid and on-site assay for detection of gene and allelic differentiation in an organism and can replace existing diagnostic assays.

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