Research Article

Development and Evaluation of Loop Mediated Isothermal Amplification Assay for the Detection of Pathogens

A. Ragavee, S. Asha Devi*
School of Bio Sciences and Technology, VIT University, Tamil Nadu, India.
***Corresponding author's E-mail:** ashaselvaraj74@gmail.com

Accepted on: 13-06-2015; **Finalized on:** 31-07-2015.

ABSTRACT

A Loop mediated isothermal AMPlification (LAMP) assay with three pairs of primers targeting a highly conserved region of gene of interest is developed to diagnose the pathogens. The LAMP has emerged has an alternative diagnostic tool to PCR for the detection of pathogens. The reaction time of LAMP assay is shortened to 30 min after optimizing the reaction system. The developed technique is compared to that of the conventional PCR. Thus established LAMP assay will play an important role in the rapid and specific detection.

Keywords: LAMP, Diagnostic tool, Pathogens, PCR.

INTRODUCTION

Nucleic acid amplification is one of the most valuable tools in virtually all life science fields, including application-oriented fields such as clinical medicine, in which diagnosis of infectious diseases, genetic disorders and genetic traits are particularly benefited by this new technique. In addition to the widely used PCR-based detection several amplification methods have been invented.[1] They include nucleic acid sequence–based amplification (NASBA)[2], self-sustained sequence replication (SR)[3] and Strand Displacement Amplification (SDA)[4,5]. Each of these amplification methods has its own innovation to re-initiate new rounds of DNA synthesis. For example, PCR uses heat denaturation of double-stranded DNA products to promote the next round of DNA synthesis. SR and NASBA eliminate heat denaturation by using a set of transcription and reverse transcription reaction to amplify the target sequence. Similarly, SDA eliminates the heat denaturation step in cycling DNA synthesis by employing a set of restriction enzyme digestions and strand displacement DNA synthesis with modified nucleotides as substrate.

These methods can amplify target nucleic acids to a similar magnitude; all with detection limit of less than 10 copies and within an hour or so, but still have shortcomings to overcome[6,7]. They require either a precision instrument for amplification or an elaborate method for detection of the amplified product due to poor specificity of target sequence selection. Despite the simplicity and the obtainable magnitude of amplification, the requirement for a high precision thermal cycler in PCR prevents this powerful method from being widely used, such as in private clinics as a routine diagnostic tool. On the other hand, NASBA and SR, which do not use thermal cycling, are compromised in specificity, resulting mainly from the necessity to use a relatively low temperature of 40°C for amplification. SDA largely overcomes these shortcomings by using six primers and isothermal conditions for amplification, but still has weak points: increased backgrounds due to digestion of irrelevant DNA contained in the sample and the necessity to use costly modified primers, such as in nested PCR and SDA, has improved amplification specificity for the target sequence, residual co-amplification of irrelevant sequences still causes a general setback in nucleic acid amplification, particular for diagnostic use.

Loop-Mediated Isothermal Amplification

Loop-mediated isothermal amplification (LAMP) is one of the DNA amplification technology that employ a constant temperature[8]. The *Bst* polymerase plays a key role in the LAMP reaction process. The *Bst* polymerase, which is derived from *Bacillus stearothermophilus* living in hot springs with temperature around 70°C, has polymerize activity, 5′-3′ exonuclease activity, and strand displacement ability. At a suitable temperature, *Bst* polymerase with strand displacement activity can separate the non-template strand from the template DNA without the thermal cycles of the PCR process, in which *Taq* polymerase is used to synthesize new DNA strands. Subtle primer design is also necessary for a successful LAMP reaction. In the first stage of the reaction, the so-called outer and inner primer pairs can make dumbbell-like loop DNA strands from the target DNA templates, and the dumbbell-like DNA strands become the new template DNA for the next step. The dumbbell-like DNA strands then continue replicating to become a flower-like long-chain DNA product[9]. In addition to these two primer pairs, a third pair known as loop primers has been designed and proven to be beneficial in accelerating the amplification process. A good primer design not only ensures successful execution of LAMP, but also increases the sensitivity and specificity of the reaction result[10]. Thus, the LAMP reaction is carried out by three pairs of primers in an isothermal condition. Compared to the PCR, the reaction time of LAMP is shorter while the sensitivity

and specificity are almost the same or even better. For fixed temperature heating, the heater component of the device can be simpler relative to traditional DNA amplification instruments. These features afford LAMP strong potential as a disease screening method based on the economic benefits of clinical point-of-care devices with simpler designs. Because of convenience, high efficiency, and the specificity of LAMP, it has been applied to many DNA screening tests.

The invention of the Loop-mediated isothermal AMPlification (LAMP) method in a decade ago has given new impetus towards development of point of care diagnostic tests based on amplification of pathogenic DNA, a technology that has been the precinct of well-developed laboratories. The LAMP technology amplifies DNA with high sensitivity relying on an enzyme with strand displacement activity under isothermal conditions. Additionally, the technology uses four to six specially designed primers recognising six to eight regions of the target DNA sequence, hence a high specificity. The auto-cycling reactions lead to accumulation of a large amount of the target DNA and other reaction by-products, such as magnesium pyrophosphate, that allow rapid detection using varied formats[8,11]. Over the last 10 years, LAMP has been used widely in the laboratory setting to detect pathogens of medical and veterinary importance, plant parasitic diseases, genetically modified products, and tumour and embryo sex identification, among other uses[12]. However, its application under field conditions has been limited, partly due to the infancy of the technologies associated with LAMP, such as field-based template preparation methods and product detection formats. In this Viewpoint, it highlights the essential technologies that require development before the LAMP platform can be progressed into a realistic point of care format for resource-poor endemic areas.

Assured Tests

Lack of effective point of care diagnostic tests applicable in resource-poor endemic areas is a critical barrier to effective treatment and control of infectious diseases. Indeed, this paucity is acutely demonstrated in Neglected Tropical Diseases (NTDs), where access to reliable diagnostic testing is severely limited and misdiagnosis commonly occurs. Although the reasons for the failure to prevent and control NTDs in the developing world are complex, a major barrier to effective health care is the lack of access to reliable diagnostic laboratory services[13]. The World Health Organization (WHO) recommends that an ideal diagnostic test suitable for developing countries should be affordable, sensitive, specific, user-friendly (simple to perform in a few steps with minimal training), robust and rapid (results available in 30 min), equipment free, and deliverable to the end user[14]. So far, only a few Rapid Diagnostic Test (RDT) formats fit this model, albeit with limited sensitivity and specificity[15]. Nucleic acid (DNA) amplification tests targeting pathogen markers have high sensitivity and specificity but generally fail to meet the assured guidelines in terms of affordability, rapidity, and being equipment free[16]. However, with the recent invention and advancement of isothermal technologies[1], development of assured tests based on DNA amplification seems realistic. One such potential method is the LAMP technology, which has salient advantages over most DNA-based amplification tests. These characteristics make LAMP strategy a potential assured platform. However, for LAMP technology to demonstrate the performance goals implied in the assured guidelines, four technologies that are applicable with the LAMP test need to be developed. These include template preparation protocols, a lyophilised kit, a reliable power source and product detection technologies.

MATERIALS AND METHODS

Samples

The clinical or environmental samples can be used. The samples must be processed and used for the study and screened for bacteria of our interest. The samples were subjected to various preliminary screening methods.

Template Preparation

The LAMP method has the advantage of amplifying the target DNA from partially processed and/or non-processed samples[16].

This inherent advantage of LAMP shortens the reaction time and eliminates the need for DNA extraction, a step that is prone to contamination and may result in significant loss of DNA. The preparation of template DNA is the least developed method associated with LAMP technology. For example, the ideal specimens (biological fluids, tissue, swabs, scraping etc.) for LAMP reactions are yet to be determined.

The direct use of native cerebrospinal fluid, serum, heat-treated blood[17] and addition of detergent[18] have yielded viable DNA templates; however, precise preparation protocols need to be defined and optimised. Nevertheless, these results offer exciting possibilities that definition of a simple field-based template preparation protocol is possible. To improve the performance of LAMP tests, methodologies for specimen collection and processing need to be simple, optimised, and ensure high target yields. In addition, selected buffers should not only stabilise the DNA (reduce degradation in case of storage), but should also enhance amplification whenever possible. An ideal template protocol will thus depend on the sample being tested.

For example, whole blood, stool and tissue specimens may include direct boiling followed by a single buffer-purification step to separate the template from the debris or application of a sample preparation kit that readily removes unwanted biological products followed by the collection and concentration of the template. Such developments should be approached with the primary aim of reducing cost and potential LAMP inhibitors.

Primer Designing

Gene specific primers were designed based on the sequence information available in the public databases and Primer Explorer V4 Software. Primer designing is done in such a way that its length is 20 nucleotides and G+C content should be about 50-60%.

The template sequences were downloaded in FASTA format which was then aligned to find the conserved sequences. In order to avoid primer dimers synthesis the primers are constructed such that 3' end of primers were not complementary to each other. Thus a careful primer selection was done.

PCR Amplification

Reactions has to be performed in a final volume of 50 µl, template DNA, 10X PCR buffer (10 mMTris-HCl pH9.0,50 mMKCl,1.5 mM $MgCl_2$), dNTPs and respective forward and reverse primers, *Taq* DNA polymerase and nuclease free water. Amplification were performed in Programmable thermal cycler.

The annealing temperature for the PCR reaction will vary depending on the primers used for amplification.

LAMP Amplification

The LAMP reactions were carried in a 25µl or 50µl reaction mixtures containing the following reagents: thermopol reaction buffer, $MgSO_4$, Betaine, deoxynucleotide triphosphate (dNTPs) and *Bst*DNA polymerase.

The three pairs of primer are needed Forward Inner Primer (FIP), Backward Inner Primer (BIP), Forward Loop Primer (FLP), Backward Loop Primer (BLP), Forward Outer Primer (FOP) and Backward Outer Primer (BOP). Finally an appropriate amount of template genomic DNA were added to the reaction tube.

The reaction were carried out in a water-bath at 65°C for 30 min and inactivated at 80°C for 5 min in heat-block.

Detection of LAMP

Significant progress has been made with modification of the LAMP method, but the product detection technologies have not seen similar advancement.

The LAMP reaction produces large amounts of magnesium pyrophosphate (a white precipitate) and dsDNA, which allow visual inspection of results using a turbid meter and real-time PCR machine, respectively, avoiding post DNA amplification manipulation.

To achieve a white precipitate, extra Mg^{++} is required, which may compromise test specificity[19], while fluorescence dyes have a major limitation in that they can bind non-specifically to any dsDNA, such as primer-dimers, leading to erroneous result interpretation.

Other reported non-specific dyes (Table.1)[20] include calcein and hydroxynaphthol blue, which are incubated with the reaction, and SYBR Green I, which is added after the reaction, a step that may lead to subsequent contamination once the tubes are opened.

A higher test specificity can be achieved by targeting an internal sequence of the amplicon through incorporating fluorescent molecular beacon probe, thus minimising non-specific signal[21], or by using a lateral flow dipstick (LFD) format (Long), though at a slightly higher cost. In general, the use of dyes is most preferred because they are cheap and most allow definite visual inspection of results based on colour change[22].

However, the need for treatment and/or a decision on case management dictates unequivocal result interpretation.

Thus, any LAMP detection format should not compromise specificity for simplicity. An ideal LAMP detection format would thus include a closed amplification and detection unit to limit contamination and use of a dye of acceptable specificity and/or a novel LFD format.

CONCLUSION

The LAMP reactions can be performed and its products were detected without electrophoresis using ambient light after the addition of a number of DNA dyes to the reaction tube.

The dyes include SYBR green, ethidium bromide, hydroxynaphthol blue, Magnesium pyrophosphate, propodium iodide.

This is a rapid and economical assay to detect the gene of interest which makes this assay amenable to point of care.

There are several advantages of the LAMP it is conventional method for DNA amplification. This assay only needs a heating block and obviates the need for a thermal cycler. Another advantage of this assay is that identification of a positive reaction does not require any special processing or electrophoresis. It can be detected by looking for a colour change of the reaction mix in ambient light, when a DNA-binding dye is used. This visualization can be further enhanced by UV trans-illumination.

This assay is suited for qualitative but not quantitative information.

As such sensitive diagnostic tests are required to guide treatment, minimize drug exposure to non-infected individuals and interrupt disease transmission.

Considering LAMP's simplicity in operation and high sensitivity, there is potential use in clinical diagnosis and surveillance of infectious diseases.

This can be used as effective tool for the detection of gene of interest and it facilitate the provision of a same day testing strategy in even the most remote rural health facilities.

Table 1: DNA-binding dyes and their interpretation

Dyes	Detection Method	Safety	Positive Reaction	Negative Reaction	Need For UV Rays	Stability	Cross Contamination	Use Time
Magnesium Pyrophosphate	Visual	Yes	Turbidity	No turbidity	No	5-10 Sec	No	Before amplification
Ethidium Bromide	Visual	No	Yellow	No colour	Yes	2 weeks	Yes	After amplification
SYBR Green	Visual	Yes	Green	Red	Yes	1-3 days	Yes	After amplification
Hydroxynaphthol blue	Visual	Yes	Sky Blue	Violet	No	2-3 weeks	No	Before amplification
Genefinder	Visual	Yes	Green	Red	No	2-3 weeks	No	Before amplification

REFERENCES

1. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T, Loop Mediated Isothermal Amplification of DNA. Nucleic Acids Research, 28, 2000, e63.
2. Haug A, Gjevre AG, Skjerve E, Kaldhusdal M. A survey of the economic impact of subclinical Eimeria infections in broiler chickens in Norway. Avian Pathology, 37, 2008, 333-341.
3. Williams, RB. A compartmentalised model for the estimation of the cost of coccidiosis to the world's chicken production industry. International Journal of Parasitology, 29, 1999, 1209-1229.
4. Chapman H, Cherry T, Danforth HD, Richards G, Shirley MW, Williams RB. Sustainable coccidiosis control in poultry production: the role of live vaccines. International Journal of Parasitology, 32, 2002, 617-629.
5. McDougald LR, Fuller L, Mattiello R. A survey of coccidia on 43 poultry farms in Argentina. Avian Diseases, 41, 1997, 923-927.
6. Williams RB, Marshall RN, Pages M, Dardi M, DelCacho E. Pathogenesis of Eimeria praecox in chickens: virulence of field strains compared with laboratory strains of Eimeria praecox and Eimeria acervulina. Avian Pathology, 38, 2009, 359-366.
7. Johnson J, Malcolm Reid W. Anticoccidial drugs: lesion scoring techniques in battery and floor-pen experiments with chickens. Experimental Parasitology, 28, 1970, 30-36.
8. Fernandez S, Katsuyama AM, Kashiwabara AY, Madeira AM, Durham AM, Gruber A. Characterization of SCAR markers of Eimeria spp. of domestic fowl and construction of a public relational database (The Eimeria SCARdb).FEMS Microbiology Letters, 238, 2004, 183-188.
9. Beck HP, Blake D, Darde ML,. Felger I, Pedraza-Diaz S, Regidor-Cerrillo J, Gomez-Bautista M, Ortega-Mora LM, Putignani L, Shiels B. Molecular approaches to diversity of populations of apicomplexan parasites. International Journal of Parasitology, 39, 2009, 175-189.
10. Xu J, Rong R, Zhang HQ, Shi CJ, Zhu XQ, Xia CM. Sensitive and rapid detection of Schistosoma japonicum DNA by loop-mediated isothermal amplification (LAMP). International Journal of Parasitology, 40, 2010, 327-331.
11. He L, Zhou YQ, Oosthuizen MC, Zhao JL. Loop-mediated isothermal amplification (LAMP) detection of Babesia orientalis in water buffalo (Bubalus babalis, Linnaeus, 1758) in China. Veterinary Parasitology, 165, 2009, 36-40.
12. FAO/EBRD. Agribusiness Handbook: Poultry Meat & Eggs., Rome 2010: FAO.
13. Perry BD, Randolph T.F, Mc Dermott JJ, Sones KR,. Thornton PK. Investing in animal health research to alleviate poverty. International Livestock Research Institute. Nairobi, Kenya, 2002, 148 pp.
14. Thekisoe OM, Rambritch NE, Nakao R, Bazie RS,. Mbati P, Namangala B, Malele I, Skilton RA, Jongejan F, Sugimoto C, Kawazu S, Inoue N. Loop-mediated isothermal amplification (LAMP) assays for detection of Theileria parva infections targeting the PIM and p150 genes. International Journal of Parasitology, 40, 2010, 55-61.
15. Nagamine K, Hase T, Notomi T. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Molecular Cellular Probes, 16, 2002, 223-229.
16. Wastling SL, Picozzi K, Kakembo AS. Welburn SC. LAMP for human African trypanosomiasis: a comparative study of detection formats. PLOS Neglected Tropical Diseases, 4, 2010, e865.
17. Goto M, Honda E, Ogura A, Nomoto A, Hanaki K. Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxynaphthol blue. Biotechniques, 46, 2009, 167-172.
18. Karanis P, Thekisoe O, Kiouptsi K, Ongerth J, Igarashi I, Inoue N. Development and preliminary evaluation of a loop-mediated isothermal amplification procedure for sensitive detection of Cryptosporidium oocysts in fecal and water samples. Applied and Environmental Microbiology, 73, 2007, 5660-5662.
19. Long P, Joyner L, Millard B, Norton C. A guide to laboratory techniques used in the study and diagnosis of avian coccidiosis. Folia Veterinaria Latina, 6, 1976, 201-217.
20. Mohammad AA, Ojaghkandi MA, Hemmatabadi A, Hamidi F, Aghaei S. Development of Colorimetric Loop-Mediated Isothermal Amplification Assay for Rapid Detection of the Tomato Yellow Leaf Curl Virus. Plant Pathology and Microbiology, 4, 2013, 153.
21. Walsh SP, Metzger DA, Higuchi R. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques, 10, 1991, 506-513.
22. Rozen S, Skaletsky H. Primer on the WWW for general users and for biologist programmers. Methods in Molecular Biology, 132, 2000, 365-386.

Source of Support: Nil, **Conflict of Interest:** None.