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Review

Loop-mediated isothermal amplification (LAMP) based detection of bacteria: A Review

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Various diseases are caused by pathogenic bacteria and their diagnosis depends on accurate detection of pathogen from clinical samples. Several molecular methods have been developed including PCR, Real Time PCR or multiplex PCR which detects the pathogen accurately. However, every method has some limitations like low detection limit, whereas Loop-mediated isothermal amplification (LAMP) is a powerful and novel nucleic acid amplification method, which detects the DNA at very low level compared to other methods. This method amplifies very few copies of target DNA with high specificity, efficiency and rapidity under isothermal conditions by using a set of four specially designed primers and a DNA polymerase with strand displacement activity. This review presents detection of various bacteria by LAMP method and covers their detection limit in clinical specimens.

Key words: Bacteria, Loop-mediated isothermal amplification (LAMP), sensitive, rapid, simple.

INTRODUCTION

Isolation and characterization of pathogens from clinical samples is a tedious job. Traditional methods of microbial identification rely on the phenotypic characteristics like bacterial fermentation, fungal conidiogenesis, parasitic morphology, and viral cytopathic effects which are commonly used. Some phenotypic characteristics are sensitive enough for strain characterization; these include biotyping, isoenzyme profiles, antibiotic susceptibility profiles, and chromatographic analysis of cellular fatty acids (Pierson et al., 1992; Blanc et al., 1994; Stoakes et al., 1994; Thurm and Gericke, 1994; Lin et al., 1995). Advances in molecular biology over the past 10 years have opened new areas for microbial identification and characterization (Erlich et al., 1991; Mullis and Faloona, 1987; Persing, 1991; Saiki et al., 1988). Molecular biology techniques (for characterization of specific genes or gene segments) are now common in the clinical laboratories.

Brucella spp. are facultative intracellular bacteria that cause zoonotic disease of brucellosis worldwide to humans and animals (that is, cattle, goats, and pigs) leading to economic losses for the livestock industry. Detection of *Brucella* spp. takes 48 to 72 h (Kumar et al., 1997; Barrouin-Melo et al., 2007) that does not meet the rapid detection requirement of food industries. Due to the urgent need of fast, specific, sensitive and inexpensive

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method for the diagnosis of *Brucella* spp., Chen et al. (2013) developed LAMP method for its detection. According to WHO report, tuberculosis (TB) is second leading cause of death among infectious diseases worldwide after the human immunodeficiency virus (HIV) (WHO, "Global Tuberculosis Report," 2012). *Mycobacterium tuberculosis* is a slow-growing bacterium that needs 1 to 2 months for growing in a culture. Therefore, to control TB, a rapid and timely diagnosis of tuberculosis is essential to combat this disease. Kaewphinit et al. (2013) developed LAMP method for detection of *M. tuberculosis* bacteria from clinical sputum samples. Due to their rapidity and high sensitivity, such advanced molecular methods improve clinician's ability to interpret test results which further enable them to better customize their patient care. There are many articles covering importance of LAMP method as an effective diagnostic tool for infectious diseases (Notomi et al., 2000; Mori and Notomi, 2009; Fakruddin 2011; Saharan et al., 2014). This review is planned to study about the details of pathogenic bacteria detected by LAMP method.

WHY THERE IS NEED OF LAMP

Earlier, DNA hybridization studies were used to demonstrate relatedness among different bacteria. These technologies, however, like the determinations of phenotypic variables, are limited by microbial recovery and growth but then after few years, diagnostics using DNA-based tools, such as polymerase chain reaction (PCR), are increasingly popular due to their specificity and speed, as compared to culture-based methods (Louie et al., 2000). The amplified products, known as amplicons, may be characterized by various methods, including nucleic acid probe hybridization, analysis of fragments after restriction endonuclease digestion, or direct sequence analysis (Persing, 1991; Wagar, 2006). Further variations of PCR method like RT- PCR, ligase chain reaction (LCR), nested PCR, and multiplex PCR, etc have simplified and accelerated the process of nucleic acid amplification and easy detection of microbes (Wagar, 2006) but these all have drawbacks of less sensitivity, insufficient specificity, low amplification efficiency, not available for all species, high cost, use of special equipments etc. that is thermo cycler, complicated result detection methods, etc. So, there is a need of another powerful technique which can overcome all these drawbacks and this all became possible with LAMP.

Although the inception of loop-mediated isothermal amplification (LAMP) refers back to 1998, the popularity of LAMP starts only after 2003 following emergence of West Nile and SARS viruses. This technique was first described and initially evaluated for detection of hepatitis B virus DNA by Notomi et al. (2000). First of all, LAMP has been applied to many kinds of pathogens causing food-borne diseases (Lukinmaa et al., 2004). LAMP kits for detecting *Salmonella*, *Legionella*, *Listeria*, verotoxin-producing *Escherichia coli*, and *Campylobacter* have been commercialized. LAMP is a powerful and novel nucleic acid amplification method based on the principle of strand displacement activity that amplifies a few copies of target DNA with high specificity, efficiency, and rapidity under isothermal conditions, using a set of four specially designed primers and a DNA polymerase. The cycling reactions can result in the accumulation of 10^9 to 10^{10} fold copies of target in less than an hour (Notomi et al, 2000; Parida et al., 2008; Tomita et al., 2008). A large amount of product is formed, due to the strand displacement activity of *Bst* polymerase enzyme and because of this property; identification of a positive reaction does not require any special processing or electrophoresis (Mori et al., 2001). LAMP is isothermal which eradicates the need for expensive thermo cyclers used in conventional PCR; it may be a particularly useful method for infectious disease diagnosis in low and middle income countries (Macarthur, 2009).

LAMP METHODOLOGIES

Collection of bacterial strain

In LAMP method, infected blood samples from patients, infected food samples (fruit juices, various types of drinks, etc), sputum sample (in case of TB patients), urine and field samples (that is, collected directly from site of infection or from medical centers) can be used directly for detection of the pathogen.

Genomic DNA extraction from bacterial culture

There are a number of methods available that can be used to extract template for the LAMP process. These methods vary depending on the source material and whether RNA or DNA is required for the procedure. Commercial column based kits are most frequently used and have been used successfully for extraction from microbial cell cultures (En et al., 2008; Kubota et al., 2008; Tomlinson et al., 2007), animal tissue culture and from plant host species (Fukuta et al., 2003; Varga and James, 2006). However, a crude CTAB method has also been used to successfully extract the citrus greening organism from *Citrus* species (Okuda et al., 2005). Similar crude heat lysis methods have been used for many bacterial species (Savan et al., 2004; Song et al., 2005).

Design of primers for the LAMP method

DNA sequence is retrieved from NCBI (http://www.ncbi:nlm.nhi.gov/) and specific LAMP DNA oligonucleotide primers are designed from DNA sequence

using free online software that is, Primer-Explorer IV software program (http://venus.netlaboratory.com/partner/LAMP/pevl.html). The following four types of primers based on 6 distinct regions of the target gene; that is F3c, F2c and F1c regions at the 3' side and B1, B2 and B3 regions at the 5' side are to be designed:

i) F3 Primer: Forward Outer Primer consists of the F3 region that is complementary to the F3c region.
ii) B3 Primer: Backward Outer Primer consists of the B3 region that is complementary to the B3c region.
iii) FIP: Forward Inner Primer (FIP) consists of the F2 region (at the 3' end) that is complementary to the F2c region, and the same sequence as the F1c region at the 5' end.
iv) BIP: Backward Inner Primer (BIP) consists of the B2 region (at the 3' end) that is complementary to the B2c region, and the same sequence as the B1c region at the 5' end.

LAMP reaction

The LAMP reaction is carried out in a 25 µL reaction mixture containing 0.8 µM each of forward inner primer and backward inner primer, 0.2 µM each of F3 and B3, 400 µM each of deoxynucleoside triphosphate (dNTP), 1 M betaine, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 4 mM MgSO4, 0.1% Triton X-100, 8 U Bst DNA polymerase large fragment. 2 µl target DNA was added and mixture was incubated at 65°C for 1 h using a conventional heating block and then heated to 80° C for 10 min to terminate the reaction.

Mechanism of LAMP

The mechanism of the LAMP amplification reaction includes three steps: Production of starting material, cycling amplification, and recycling (Notomi et al., 2000; Tomita et al., 2008). Two inner and two outer primers are required for LAMP. In the initial steps of the LAMP reaction, all four primers are employed, but in the later cycling steps, only the inner primers are used for strand displacement DNA synthesis. The outer primers are referred to as F3 and B3, while the inner primers are forward inner primer (FIB) and backward inner primer (BIP). Both FIP and BIP contains two distinct sequences corresponding to the sense and antisense sequences of the target DNA, one for priming in the first stage and the other for self-priming in later stages (Notomi et al., 2000). The size and sequence of the primers was chosen so that their melting temperature (Tm) is between 60 and 65°C, the optimal temperature for *Bst* polymerase. The final product in LAMP is a mixture of stem loop DNA with various stem length and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target sequence in the same strand (Notomi et al., 2000; Tomita et al., 2008).

Detection of amplified products

A number of methods are available that can be used for detection of products in LAMP method. Amplified products can be directly observed by the gel electrophoresis, naked eye or using a UV trans-illuminator, intercalating dyes like SYBR Green I stain, pitco green (Dukes et al., 2006; Iwamoto et al., 2003; Tomlinson and Boonham, 2008), by-products from the reaction chemistry (Goto et al., 2009) or by addition of hydroxyl-napthol blue, a chelating agent that changes colour due to the change in the concentration of Mg^{2+} ions (Goto et al., 2009).

Detection of amplicons or LAMP products

Naked eye: LAMP products can be directly observed by the naked eye in the reaction tube by adding 2.0 µl of 10 fold diluted SYBR Green I stain.

UV transilluminator: Under UV illumination, the gel shows a ladder-like structure.

Gel Electrophoresis: The result of LAMP reactions may be detected using gel electrophoresis.

Intercalating Dyes: The high specificity product produced during the LAMP process offers the use of intercalating dyes for amplification product detection. Intercalating dyes include SYBR green and Picogreen. Both dyes can be detected visually or by measurement in a real-time PCR machine or equivalent flourometer (Dukes et al., 2006; Iwamoto et al., 2003; Tomlinson and Boonham, 2008).

Chemical reactions: Two other alternatives that is, Magnesium pyrophosphate, which increases the turbidity of the reaction by precipitation, allowing the detection visually or more commonly, by spectrophotometer (Mori et al., 2011) and another is hydroxyl-napthol blue, a chelating agent that changes colour due to the change in the concentration of Mg^{2+} ions (Goto et al., 2009).

ADVANTAGES OF LAMP

A variety of pathogenic bacterial strains like *E. faecalis*, *M. ulcerans, M. tuberculosis, M. Pneumonia, S. typhi, B. anthracis* etc. were successfully identified by LAMP method developed by various researchers shown in Table 1.

Simplicity and cost-effectiveness

1) Isothermal - no need for thermal cycler,

Table 1. List of bacteria detected by LAMP assay till date.

Author's name	Organism name	Detection limit
Xu et al., 2014	*E. faecalis*	3.2 CFU/250 ml
Su et al., 2014	*S.aureus*	10 CFU/reaction
Kaewphinit et al., 2013	*M. tuberculosis*	5 pg
Lim et al., 2013	*S. aureus*	2.5 ng/µl
Wang et al., 2012 (a)	*S. agalactiae*	$2.8x10^3$ CFU/ml
Wang et al., 2012 (b)	*E. coli* various serogroups	10^3-10^4 CFU/ g
De Souza et al., 2012	*M. ulcerans*	48pg/µl
Tang et al., 2012	*S. enteric*	6.0CFU/test
Nagarajappa et al., 2012	Enterotoxigenic *Staphylococci*	100CFU/test
Yang et al., 2012	*Borrelia burgdorferi*	0.02-0.2pg
Sun et al., 2011	*V. parahaemolytics*	$2.4x10^2$ CFU/ml (pure), $8.9x10^2$CFU/ml (infected food sample).
Han et al., 2011	*V. vulnificus*	$2.5x10^3$ CFU/g
Kubota et al., 2011	*Ralstonia solanacearum*	10^4-10^6 CFU/ml
Kohan et al., 2011	*M. tuberculosis*	5 fg/reaction
Suwanampai et al., 2011	*S. aureus*	10^4 CFU/ml
Tang et al., 2011	*Listeria monocytogenes*	2.0 CFU/reaction
Lin et al., 2011	*Chlamydia psittaci* abortus strain	25 copies
Pan et al., 2011	*Brucella species*	10 pg (pure), 1.3x103 CFU/ml (contaminated milk).
Yang et al., 2011	*S. aureus*	1.25 CFU/reaction tube (pure), 10.3CFU/reaction tube (contaminated).
Ward et al., 2010	*Xylella fastidiosa*	200-25 0copies/reaction
Xu et al., 2010	*V. cholera*	25 CFU (pure), 32CFU (infected sample)
Techathuvanan et al., 2010	*S. typhimurium*	10^2-10^6 CFU/25g
Zhao et al., 2010	*S.* species	100 CFU/reaction
Fukasawa et al., 2010	*M. tuberculosis*	5,000 bacilli/ml sputum
Lu et al., 2010	*Legionella pneumophila*	576 fg (pure), 8CFU/ml (infected water sample).
Iseki et al., 2010	*Plasmodium knowlesi*	10^2-10^8 copies/µl
Nakao et al., 2010	*Ehrlichia ruminantium*	10 copies
Rigano et al., 2010	*Xanthomonas axonopodis* pv.*citri*	10 fg (pure), 18CFU (infected).
Kawai et al., 2009	*Chlamydophila pneumonia*	100%
Gahlawat et al., 2009	*Renibacterium salmoninarium*	10^{-8}
Yamazaki et al., 2010	*Vibrio paraharmolyticus* tdh and trh genes	0.8 CFU (tdh), 21.3CFU (trh-1), 5.0 CFU(trh-2).
Li et al., 2009	*Pseudomonas syringae pv. phaseolica*	$6.9x10^3$ CFU/ml
Hill et al., 2008	*Escherichia coli*	10 copies/reaction
Salah et al., 2008	*Renibacterium salmoninarum*	1 pg
Yamazaki et al., 2008 (a)	*Campylobacter jejuni*	5.6 CFU/g
Yamazaki et al., 2008 (b)	*V. cholerae*	$7.8x10^2$ CFU/g
Pandey et al., 2008	*M. tuberculosis*	100%
Misawa et al., 2007	Methicillin-resistant *S. aureus*	92.3%
Hara-kudo et al., 2007	*E. coli*	0.7 CFU/test
Qiao et al., 2007	*B. anthracis*	10 spores/tube (pure), 100spores/2mg powder (infected)
Boehme et al., 2007	Pulmonary tuberculosis	97.7%
Kato et al., 2007	*E. faecalis*	10 µg/tube
Aoi et al., 2006	Ammonia-oxidizing bacteri a	10^2 copies
El-Matbouli et al., 2006	*Thelohania contejeani*	10^{-5}
Kamachi et al., 2006	*Bordetella pertussis*	10 fg/DNA tube
Yeh et al., 2006	*Flavobacterium columnare*	30 pg/reaction tube
Mukai et al., 2006	*M. species*	500 copies

Table 1. Contd.

Ohtsuka et al., 2005	*S.enteric*	92.3%
Kato et al., 2005	*Clostridium difficile*	50 ng-0.5pg
Savan et al., 2005	Fish and shellfish pathogens	20 CFU
Hara-Kudo et al., 2005	*Salmonella*	2.2 CFU/test
Saito et al., 2005	*Mycoplasma pneumonia*	$2x10^2$ copies
Yeh et al., 2005	*Edwardsiella ictaluri*	20 CFU/ml
El-Matbouli et al., 2005	*Tetracapsuloides bryosalmonae*	100 folds more sensitive
Yoshida et al., 2005	*Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola*	1mg/tube(*P.gingivalis*),100fg/tube(*T.forsythia*),1mg/tube (*T. denticola*).
Seki et al., 2005	*S.. pneumonia*	10 copies
Maeda et al., 2005	*Porphyromonas gingivalis*	10^2-10^6 cells
Song et al., 2005	*Shigella* and enteroinvasive *Escherichia coli*	8 CFU/reaction
Horisaka et al., 2004	*Yersinia pseudotuberculosis*	10 CFU
Savan et al., 2004	Edwardsiellosis	$3.8X10^2$ CFU
Enosawa et al., 2003	*M. avium* subsp. para-tuberculosis	0.5-5 pg/tube
Iwamoto et al., 2003	*M. tuberculosis* complex	5-50 copies

g, gram; mg, milligram; ml, milliliter; pg, picogram; ng, nanogram; µl, microliter; µg, microgram; fg, femtogram; CFU, colony forming unit.

2) All required reagents are relatively cheap,
3) No need for excessive post-reaction handling steps.

Specificity

The use of six primers in LAMP provides a greater specificity than PCR. LAMP is less susceptible to interference (Notomi et al., 2000). LAMP is more specific than other techniques as many researchers have achieved even 100% specificity (Misawa et al., 2007; Tao et al., 2011; Wang et al., 2012; Yamazaki et al., 2008; Wang et al., 2010; Zhao et al., 2010) 97.3% specificity (Yeh et al., 2006), 95.9% (Pandey et al., 2008) and 94.2% (kohan et al., 2011) specificity.

Sensitivity

Many researchers have reported of achieving LAMP sensitivity as low as 6 copies/reaction for pure template. There is a general consensus that LAMP is 10 times more sensitive than standard PCR (En et al., 2008; Fukuta et al., 2003; Okuda et al., 2005; Savan et al., 2004; Dukes et al., 2006; Tomlinson and Boonham, 2008).

Rapidity

As the PCR and other methods proved to be time consuming, LAMP method is very fast and rapid. It can detect the infected bacteria; that is, generate results in an average of half an hour.

Direct use of sample from site of infection

In PCR and other molecular techniques for detection of pathogens, nucleic acid needs to be isolated but due to LAMP, it became possible to use directly the infected blood sample, food sample, sputum, urine samples directly from the site of infection. When compared to PCR, LAMP proves better than PCR in many ways as shown in Table 3. From all these, we can conclude that LAMP is a fast, rapid, economic, versatile and very valuable method and have emerged as a new era in the field of technology.

Lamp detection kits

Till now, a large number of bacterial pathogens have been detected by LAMP and still the research is going on but, after the detection of bacteria, some researchers have developed ready-made kits (Table 2) for more rapid and easier detection to be used at commercial level. These kits have all the reagents (thermopol buffer, betaine, dNTP's, primers, Bst polymerase enzyme, $MgSO_4$ in appropriate concentration) in it except, the nucleic acid sample which has to be added at the time of need. These ready-made kits have been commercialized by Eiken chemical company for detection of *M. tuberculosis* and Campylobacter spp. etc. (Eiken Chemical Co., Ltd. (Head office in Taito-ku, Tokyo).

SUMMARY AND FUTURE ASPECTS

No need for denaturing step in using the LAMP method. The whole amplification reaction takes place continuously

Table 2. LAMP based commercially available bacterial pathogen detection kits are listed below.

Year	Organism name	Name of kit
Mitarai et al., 2011	*M. tuberculosis*	Evaluation of a simple loop-mediated isothermal amplification test kit for the diagnosis of tuberculosis.
Eiken Chemical Co., Ltd., 2011	*M. tuberculosis*	Release of the “Loopamp® Tuberculosis Complex Detection Reagent Kit”, a pharmaceutical for *in vitro* diagnosis, as well as the “Loopamp® PURE DNA Extraction Kit”, “Loopamp® LF-160 Homeothermal Equipment with Fluorometer” and “PureLAMPTM Heater”
Eiken Chemical Co., Ltd., 2008	*M. tuberculosis*	“Loopamp TB detection Kit”
Eiken Chemical Co., Ltd., 2008	*C., Giardia*	Loopamp Cryptosporidium Detection Kit" and "Loopamp Giardia Detection Kit".
Eiken Chemical Co., Ltd., 2006	*Campylobacter*	"Loopamp *Campylobacter* detection Kit".
Eiken Chemical Co., Ltd., 2005	*E.coli O157*	Loopamp O157 detection Kit".
Eiken Chemical Co., Ltd., 2005	*L. monocytogenes*	Loopamp *L. monocytogenes* detection Kit".
Eiken Chemical Co., Ltd., 2004	*Legionella*	Loopamp *Legionella* screening Kit E" for environmental detection.
Eiken Chemical Co., Ltd., 2003	*Salmonella,* verotoxins	Novel Loopamp *Salmonella* screening kit, Loopamp verotoxin-producing *Escherichia coli* screening kit, and Loopamp Verotoxin Typing Kit.

Table 3. Comparison of PCR and LAMP.

Difference	PCR	LAMP
Definition	PCR is a rapid and simple technique of producing relatively large numbers of copies of DNA molecules from minute quantities of source DNA material	Loop-mediated isothermal amplification (LAMP) that amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions
Denaturation step	Denaturation step is compulsory: Denature double stranded into a single stranded form	No need for a step to denature double stranded into a single stranded form
Specificity	Two primers are to amplify template DNA.	Four specially designed primers that recognize a total of six distinct sequences on the target DNA
Sensitivity	The sensitivity and specificity are not 100%	The sensitivity and specificity are 100%
Time requirement	PCR take more time than LAMP	LAMP take less time than PCR
Cost	Costly method in comparison to LAMP (5–7 $US per sample)	Cheapest method in comparison to PCR (about 70 cents US per sample)

under isothermal conditions. The amplification efficiency is extremely high. By designing 4 primers to recognize 6 distinct regions, the LAMP method is able to specifically amplify the target gene. The total cost can be reduced, as LAMP does not require special reagents or sophisticated equipments. The amplified products have a structure consisting of alternately inverted repeats of the target sequence on the same strand. Amplification can be done with RNA templates following the same procedure as with DNA templates, simply through the addition of reverse transcriptase.

LAMP method paves a new way to diagnose pathogenic microorganisms in clinical laboratories. It is compulsory to employ LAMP technique on large scale in resource-limited laboratories in developing countries, where many fatal tropical diseases are endemic. Also in near future, LAMP testing kits on readymade microchips are to be used by both developed and developing countries.

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