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Loop-mediated isothermal amplification: Beyond microbial identification

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Abstract: Loop-mediated isothermal amplification (LAMP) assay was introduced in the year 2000 by Notomi, as a highly sensitive, specific, and cost-effective technique for microbial identification. LAMP, a simple DNA amplification technique, with its field-amenable nature has been used to detect a variety of pathogens including viruses, fungi, bacteria, and parasites and in most of the cases it surpasses polymerase chain reaction. However, literature world has seen different set of research articles surfacing in last 5–6 years which are good example for thinking out of box. This review is the summation of selected LAMP assays which are used for different purposes other than microbial detection. This is an effort to provide a brief idea about how a small innovation to already established technique in one field can help other fields too. This review is a rundown of all the LAMP assays reported so far other than the ones which are reported for the identification of microbes. These include the implementation of LAMP assay in the field of molecular diagnosis of cancer, identification of genetically modified organisms, detection of food adulteration, eutrophication, food allergens, pesticides, identification of medicinal plants, drug resistance, and DNA methylation studies.

Subjects: Food Analysis; Fruit & Vegetables; Nutraceuticals Functional Foods

Keywords: drug resistance; food adulteration; genetically modified organisms (GMOs); loop-mediated isothermal amplification (LAMP); molecular diagnosis; polymerase chain reaction (PCR)

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PUBLIC INTEREST STATEMENT

Loop-mediated isothermal amplification (LAMP), a simple DNA amplification technique, with its field-amenable nature has been used to detect a variety of pathogens including viruses, fungi, bacteria, and parasites and in most of the cases it surpasses polymerase chain reaction (PCR). This review is a rundown of all the LAMP assays reported so far other than the ones which are reported for the identification of microbes. These include the implementation of LAMP assay in the field of molecular diagnosis of cancer, identification of GMOs, detection of food adulteration, eutrophication, food allergens, pesticides, identification of medicinal plants, drug resistance, and DNA methylation studies. The challenges faced by the available technologies are mostly the cost of the kits and their use as standalone technologies, and hence LAMP holds a promise of being affordable. We believe this would help the researchers to consider the use of this technology as an affordable and viable option.

1. Introduction

"LAMP" which stands for loop-mediated isothermal amplification shows great potential for field use as it is simple and field-amenable. This technique was developed by Notomi and co-workers in 2000. In LAMP assay, the DNA was amplified under single temperature, which bypasses the use of sophisticated and expensive thermal cyclers. The whole LAMP assay works on the synthesis of DNA through the mechanism of auto cycling and strand displacement. To perform this function, a special DNA polymerase isolated from *Bacillus stearothermophilus* (Bst) and a set of six primers which includes two loop primers specially designed to bind to unique sites on the target sequence were used. The added advantage of LAMP assay is the overall reaction completes within 30 min and an hour. During this assay, a large amount of white magnesium pyrophosphate precipitate will generate for positive results, which allows the presence of test DNA to be easily identified by visual inspection, and the positive amplification can be viewed by adding the fluorescent dyes, such as SYBR Green I (Mori, Nagamine, Tomita, & Notomi, 2001; Notomi et al., 2000).

Table 1. Applications of LAMP assay in various fields

Purpose	Detection	Gene	References
GMO	Maize	35S Promoter	Zahradnik, Kolm, et al. (2014)
	Maize	cry2Ab and cry3A	Li et al. (2014)
	Maize (T25)	Pat	Xu et al. (2013)
	Maize	Pat	Chen, Huang, Zhang, Yu, and Wu (2011)
	Rice (TT51-1)	Sucrose phosphate synthase	Chen et al. (2014)
	Rice	cry1ab	Li et al. (2013)
	Rice	Phospholipase D	Chen et al. (2012)
		Junction of KMD1 and TT51-1	
	Soybean	Lectin, Nos:	Di et al. (2014)
		GTS 40-3-2	
		MON89788	
	Soybean	3′ Junction of GTS 40-3-2	Guan, Guo, Shen, Yang, and Zhang (2010)
		3′ Junction of MON89788	
		Lectin	
	Wheat (B73-6-1)	B73-6-1	Cheng et al. (2014)
Diagnosis	Lymph node metastasis in lung cancer	Carcinoembryonic antigen-mRNA	Maeda et al. (2009)
	Neoplasm (myeloproliferative neoplasm)	JAK2V617F	Minnucci et al. (2012)
	Gastric cancer cells	Cytokeratin-19	Yoneda et al. (2014)
Allergen	Celery (*Apium graveolens*)	Mannitol dehydrogenase	Zahradnik, Martzy, et al. (2014)
Pesticide	Organophosphorus in agroproducts	Monoclonal antibody against OP	Hua et al. (2014)
Medicinal plants	Ginger (*Zingiber officinale*)	RAPD amplicon	Chaudhary et al. (2014)
Adulteration	Ostrich meat	Cytochrome b	Abdulmawjood et al. (2014)
Eutrophication	Microcystin	mcyE	Zhu et al. (2014)
Drug resistance	Multidrug resistance gene	NDM-126590244	Qi et al. (2012)
		Cfr	Qi, Du, Zhu, Zhu, and Bai (2012)
Species and sex identification	Formosa landlocked salmon	Growth hormone GH 1 and OtY2m; GU181208	Hsu et al. (2011)
Epigenetic study	Hypermethylated DNA	Promoters of CDKN2A, GATA5 and DAPK1	Zerilli et al. (2010)

Notes: GMO: genetically modified organisms, cry2Ab: crystal protein, NDM-1: New Delhi Metallo-lactamase 1, cfr: chloramphenicol-florfenicol resistance, CDKN2A: cyclin-dependent kinase inhibitor 2A, GATA5: GATA binding protein 5, DAPK1: death-associated protein kinase1.

Authors put an effort to sum up all LAMP assay articles excluding the articles where LAMP assay was developed to detect any micro-organism. This review is an attempt to make reader think out of box that LAMP assay is not only for microbial detection but can also be successfully employed for the identification of genetically modified organisms (GMOs), cancer cells, food adulterations, molecular diagnosis, drug resistance, identification of medicinal plants, and allergens to name a few (see Table 1). Moreover, all assays were found to have better sensitivity and specificity as compared to its counterpart i.e. polymerase chain reaction (PCR).

The literature search was carried out in J-GATE plus (http://jgateplus.com/search/search/?q=LAMP+assay) under the name "LAMP assay". As on 18 August 2015, there are 688 articles found containing the phrase "LAMP assay" either in title of the article or in the running material. Out of 688 articles, 28 articles were not related to microbial identification while rest of 660 articles were LAMP assays designed to identify one or the other micro-organism ranging from viruses to parasites.

LAMP, a DNA amplification technique, has been used to detect a variety of pathogens including viruses, fungi, bacteria, and parasites. Because of its simple and field-amenable nature, LAMP assay was mainly used for the detection of different microorganisms especially pathogens. On the contrary, PCR requires expensive and high-precision instruments which may not be readily available in rural endemic regions. Moreover, the Taq DNA polymerase used in PCR assay can easily be inhibited by interfering biological substances. Therefore, simple, rapid, and cost-effective detection method with high sensitivity is still needed to compensate for the limitations of PCR and other techniques. This review is the summation of all the LAMP assays reported so far except for the identification of microbes, which includes the implementation of LAMP assay in the field of molecular diagnosis of cancer, identification of GMOs, detection food adulteration, eutrophication, food allergens, pesticides, identification of medicinal plants, drug resistance, and DNA methylation study.

2. Diverse fields of application for LAMP

2.1. Molecular diagnosis

Maeda et al. (2009) developed LAMP assay for the detection of carcinoembryonic antigen-mRNA as a marker for detecting tumor cells in patients with non-small cell lung cancer. The RNAs isolated from lymph node (144 lymph nodes) and tumor (22 primary tumors) specimens were directly used for LAMP assay and compared the results with those of conventional reverse transcription–polymerase chain reaction (RT-PCR). This nodal metastasis diagnostic assay was found to be 81% sensitive and 100% specific, while the negative and positive predictive values were 91 and 100%, respectively (Maeda et al., 2009).

Minnucci et al. (2012) successfully developed the LAMP assay for the diagnosis of chronic myeloproliferative neoplasms. The myeloproliferative disorders are a group of haematological conditions at the level of the multipotent haematopoietic stem cell leading to increased production in one or more blood cell types. The three main disorders in this group are polycythaemia vera, essential thrombocythaemia and idiopathic myelofibrosis. The majority of the patients with this ailment were found harboring JAK2V617F mutation (>95%). Here a hydrophobic amino acid valine was replaced by another hydrophobic amino acid phenylalanine. Due to this, the JAK2V617F mutation has been included in the revised World Health Organization diagnostic criteria for polycythemia vera. Even though different molecular techniques are available for the detection of JAK2V617F mutation but each has its own limitations. Minnucci group developed an allele-specific LAMP assay for the detection of JAK2V617F mutation. Through LAMP assay, the group also detected low levels of mutation which were undetectable by PCR. This low tumor allele burden is important when monitoring patients treated with the aim of eradicating the disease (Minnucci et al., 2012).

In an interesting study, Yoneda, Taniguchi, Torashima, Susumu, and Kanetaka (2014) developed a RT-LAMP assay using cytokeratin 19 as a target gene for the detection of free cancer cells in

peritoneal lavage and assessed the clinical significance of the molecular diagnosis by survival analysis and frequency of recurrence with a median follow-up period of 39 months. For sensitivity evaluation of the developed assay Yoneda and group took gastric cancer MKN-45 cells which were serially diluted from 1×10^6 cells to one cell per 1×10^7 PBMCs. The mRNA was extracted from each cell fraction and RT-LAMP for CK19 mRNA was performed. As few as 10 MKN45 cells in 10^7 normal PBMCs were detected with the RT-LAMP procedures targeting CK19 mRNA using extracted mRNA of cell mixtures lysate (Yoneda et al., 2014).

2.2. Identification of GMOs

There is an increased trend of introduction of genetically modified crops in the field of agriculture that created a necessity for the development of rapid, economic, and effective on-site detection methods. Even though the PCR technique is available but it has its own drawbacks. The LAMP assay is an alternate and can be performed on site. The LAMP assay was successfully employed for the detection of different GM event and was proved to be sensitive than the routine PCR. For GMOs detection, the target for LAMP assay was mainly exogenous elements or foreign genes. More about applications of LAMP assay in the detection of GM event was discussed in the review (Li et al., 2015).

Li et al. (2015) summarized different techniques used for the screening of GMOs and specially, emphasized on LAMP assay. The review briefed about the global Status of GM Crops, GMO Safety Issues and the steps taken to regulate their introduction and listed GMOs which are successfully detected by LAMP assay and the respective targeted exogenous elements. Apart from this, the review also mentions different drawbacks associated with LAMP assay and the future perspectives with respect to the need for the use of LAMP assay for the authentication of GMOs at the same time review does not shed light on different adaptations and modification made to the native LAMP assay to make it suitable for the GMOs detection.

2.3. Food adulterations

To address the issue of authenticity of imported Ostrich meat, that is found adulterated with either beef or other less-expensive meat or with wild Ostrich species in and around Europe, Abdulmawjood et al. (2014) developed a LAMP assay and designed primers for conserved region of *cytochrome b* of mitochondrial DNA. The total DNA was isolated from 27 Ostrich samples procured from local market and through online purchase. Concurrently, they also procured reference DNA from cow, pig, sheep, goat, turkey, chicken, dog, cat, horse, and deer for identification. A set of six oligonucleotide primers were designed using LAMP Designer software, ver. 1.10 (PREMIER Biosoft, CA, USA) and for convenience they labeled as F3-Ost, B3-Ost, FIP-Ost, BIP-Ost, LoopF-Ost, and LoopB-Ost. The LAMP assay was performed without much change except a melting curve analysis at the end of the assay and the reading of results was done on a real-time fluorometer (Genie II, Optigene, UK). To check adulterated Ostrich meat in the laboratory a Swab in HYPLEX LPTV buffer test was performed. The assay showed a specificity of 100% with the investigated samples. The assay could detect as less as 1 pg of Ostrich DNA. To avoid cross contamination by aerosols, a closed system tube (Genie II) was used. The assay could detect 1 g of ostrich meat in 10 kg of meat product. Similarly, they showed the robustness of the LAMP assay by performing LAMP assay on DNA extracted from heat-treated as well as from fried meat with oil and spices gave very good results which makes a suitable assay in the investigation of food samples in restaurants or even of canned meat samples. This was the only report which addressed the issue of food adulteration with the help of LAMP assay.

2.4. Eutrophication/cynobacterial bloom

Microcystis are single-celled blue green alga, or cyanobacterium, that occurs naturally in surface waters. Microcystis can proliferate to form dense blooms and mats under certain conditions. Many variants of these cyanobacteria produce multiple toxins, including the potent liver toxin, microcystin. When Microcystis die, their cells break open, releasing the toxin microcystin into the water. Ingestion of water or algal cells containing microcystin has produced adverse effects in fish, dogs, cats, livestock, and humans. Phenotypic differentiation between toxic and nontoxic populations of Microcystis is not possible because they look similar in appearance and could coexist in a single

ecosystem. The molecular difference between these two strains was the presence of microcystin synthetase genes (mcy) which is present only in the genome of toxic strains. By considering this genotypic difference, Zhu et al. (2014) designed a LAMP assay which span around microcystin synthetase E gene (*mcyE*). Four sets of primers were designed to recognize six distinct sequences on *mcyE* gene. The protein encoded by this gene is being responsible to catalyze the addition of D-glutamate to Adda. The detection limit was found to be 8.5 pg/µl with 100% specificity. However, recent findings concluded that the presence of microcystin genes is not a useful tool for eliciting an ecological role for toxins in the environment, nor are microcystin genes (e.g. DNA) a good indicator of toxins in the environment (Beversdorf, Chaston, Miller, & McMahon, 2015). Still the use of LAMP in such a diversified field is something worth consideration.

2.5. Medicinal plants

When the agronomical characters are similar it is hard to identify inter species differences. People have addressed this issue by using classical gold standard molecular technique known as RAPD. Chaudhary and co-workers identified similar kind of problem in Zingiber genus, which includes popular medicinal plant known as ginger (Chaudhary, Khan, AlShaqha, Alharbi, & AlKhamees, 2014). There are plants which are morphological similar to ginger but their pharmacological and therapeutic properties vary. They cannot be used as a ginger for the treatment of different ailments like how the ginger and ginger extract is used. Chaudhary et al. (2014) combined RAPD and LAMP assay, and developed a marker based method for the authentication of the commercially important *Zingiber officinale* Roscoe from the closely related species. This marker based RAPD-LAMP assay could further be used by drug industry to fetch genuine phytoceuticals from different medicinal plants.

2.6. Food allergens

Celery (*Apium graveolens* L.) is a widely used ingredient in seasonings, sauces, bouillons, and instant meals. The celery consumption produced severe allergic reactions in some individuals in Europe especially in central Europe, mainly France, Switzerland, and Germany. The allergic complications include digestive disorders, respiratory distress, and skin reactions. Due to severity of celery-related allergy, the European law included celery in the lists of 14 major food allergens and must be declared in the ingredient lists whenever they appear in pre-packed food. By considering the importance of detection of celery in food products several real-time PCR assays have been developed targeting the gene Api g1. But based on the literature recommendation, Zahradnik and his group selected celery-specific mannitol dehydrogenase, and developed LAMP assay (Zahradnik, Martzy, et al., 2014). The reason behind selecting mannitol dehydrogenase as primer target was to avoid cross-reactions. For the above study, 11 plant materials including celery and 10 commercial food samples procured from Austria market were used. Interestingly, the limit of detection (LOD) for spiked food samples was found to be as low as 7.8 mg of dry celery powder per kilogram. The authors claim that the performance of the LAMP assay developed by them was found to be equal or superior to the best available PCR assay for the detection of celery in food products.

2.7. Pesticides

Organophosphorus (OP) pesticides are widely used in agriculture for the control of insect pests and equally beneficial in controlling insects that carry or transmit diseases. Over the years, the widespread use of pesticides has had several benefits and also caused many problems. OP pesticides are considered as hazardous substances because of their toxicity to nonpests and bioaccumulation and biological magnification in the environment. By considering the health hazardous problems of use of OP pesticides, Hua et al. (2014) developed iLAMP which is a rapid, sensitive, and economical method for detecting OP pesticides and their residues in food and the environment and in future can be used for the detection of other small molecules. The group used four phage-borne peptide mimotopes with specific affinities to a monoclonal antibody (mAb) against OP pesticides as a secondary reagent and came out with an iLAMP with higher sensitivity than its counterpart i.e. ELISA. One more modification made in the LAMP assay was the use of hydroxynaphthol blue as a visualization indicator in place of SYBR green so that under normal light a color change can be noticed i.e. positive sample turns violet to sky blue, so both gel electrophoresis and visualization under ultraviolet light are

omitted. The group evaluated 23 OP pesticides. One assay was used to screen eight OP pesticides with LOD between 2 and 128 ng/ml. This was the first report were LAMP assay was successfully used to detect small molecules like pesticides.

2.8. Drug resistance

Due to continuous administration of antimicrobial agents, microbes are developing resistance to these drugs. The problem of drug resistance is not confined to one place, it is a global issue and identified in broad range of micro-organisms representing itself as a serious threat to human health. The drug resistant micro-organisms are responsible for prolonged illnesses and high mortality rate. The LAMP assay is not lacking behind in addressing the serious issue like detection of drug resistance. Liu et al. (2012) successfully developed LAMP assay for the detection of New Delhi Metallo-lactamase 1 (NDM-1) carrying isolates which confer resistance to carbapenems and proved that LAMP assay was highly sensitive technique for the rapid detection of bla_{NDM-1} which confer the resistance to carbapenems. The assay was tested and conformed on pure culture, sputum, urine, and fecal samples. Similarly, Qi and co-workers developed LAMP assay for the detection of bla_{NDM-1} and another well-known antibiotic resistant gene *cfr* (chloramphenicol-florfenicol resistance) (Qi et al., 2012; Qi, Du, Zhu, Zhu, & Bai, 2012). The Cfr rRNA methyltransferase known to confers resistance to Phenicols, Lincosamides, Oxazolidinones, Pleuromutilins, and Streptogramin A antibiotics.

2.9. DNA methylation/epigenetics

It is well-established fact that tumor formation is due to the silencing of protecting guard i.e. shutting of tumor suppressor genes. The phenomenon of hypermethylation of promoter region of tumor suppressor genes is the main region behind this. By considering the importance of promoter methylation even though there are method available like methylation-sensitive restriction analysis and Methylation-specific PCR (MSP), different methylation detection kits are available by different vendors. Zerilli et al. (2010) came with novel approach which used sensitive and highly specific LAMP assay performed with three sets of methylation-specific primers. MS-LAMP was used for the detection of hypermethylated CpGs in the Promoter region of the CDKN2A (cyclin-dependent kinase inhibitor 2A), GATA5 (GATA binding protein 5), and DAPK1 (death-associated protein kinase1) genes. The study used three sets of primers specific for methylated promoters of above mentioned genes. For validation purpose, MS-LAMP assay was performed for 18 clinical tumor samples along with bisulfite-treated plasmid and genomic DNA (as controls). In order to take the assay to the next level, they grouped all the primer sets and performed a multiplex MS-LAMP for CDKN2A, GATA5, and DAPK1 and validated with proper controls. The MS-LAMP assay showed high specificity with plasmid and genomic DNA targets. The assay had a detection limit of approximately 30 copies of methylated target sequence and a selectivity of 0.5% methylated DNA in a mixture with unmethylated DNA. In MSP AT-rich bisulfite-treated DNA is more prone to generate primer dimers and nonspecific amplification, whereas MS-LAMP assay is free of primer dimers. Unless the promoter is heavily methylated, the MS-LAMP methodology may not generate a signal as the primer set may fail to detect partial methylation, that is because strict specificity of the primers. This partial methylation is sometimes present in clinical samples. The study also demonstrated the triplex MS-LAMP reaction in 2 detection formats, turbidometry and fluorescence.

2.10. Sex determination

To conserve an endangered species identification of species and its sex are the prime factors, such identification is not easy in all organisms and one such example is an endangered Formosa landlocked salmon (*Oncorhynchus masou formosanus*). As morphological differences are very minimal in salmon, Hsu and co-workers designed the LAMP assay for the same (Hsu, Hsu, Adiputra, Ohta, & Gwo, 2011). This is the first report where LAMP was successfully developed for the rapid and noninvasive identification of sex and species of this critically endangered species. LAMP primers were designed for growth hormone GH 1 gene for species identification and the male-specific marker (OtY2m; GU181208) for sex identification. The assay was optimized for as low as 0.5–5 pg of template and time is less than an hour.

3. Limitations

From the analysis done in this study it is apparent that the technique which was mainly proposed to be used for the detection of different microorganisms and was in use for this purpose for years are no more restricted to one field now. Even though LAMP is an outstanding DNA amplification procedure in which the reaction can accumulate 10^9 copies from less than 10 copies of input template within an hour, it faces some limitations. As LAMP reaction will produce high amount of products by using a little amount of DNA, the LAMP reactions should be performed carefully to avoid contamination. While the amplification reaction is extremely powerful, the quantification of LAMP product is still a challenge need to be addressed. Besides, the type of targets that LAMP can detect is also less, which to some extent limited the application of LAMP. Even though LAMP assay allow real-time detection, the signals are solely due to the accumulation of base-pairs and can easily read false amplicons as the true ones. Its powerful amplification functionality has not been well applied in the field of proteins, small molecules, Adenosine and metal ions detection areas, which present some limitations for their practical implementation. We strongly believe that the limitations will be surpassed in coming years and LAMP assay being the cost-effective and faster technique help the diagnostics globally and specially the settings with limited resources.

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Competing interests

The author declares no competing interest.

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