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LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) AND ITS VARIANTS AS SIMPLE AND COST EFFECTIVE FOR GENOTYPING METHOD

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Abstract – *Since the time of its introduction, the LAMP method has been usually used for the detection of infections in which just the amplification of the pathogen genome is required. The LAMP method is an Isothermal technique that amplifies the DNA segment of interest.*

In this review, different studies that used LAMP method as a technique for genotyping and SNPs detection were evaluated. These studies combined the LAMP method with various technologies in order to make it suitable for genotyping. Since the LAMP method is a sharp, fast and robust technique, it is suitable for use in routine diagnostics. It seems that LAMP method could be a reliable and cost-effective method for genotyping and further studies are recommended to use this approach for genotyping. In parallel with this features, new high-throughput platforms are spreading in the routine of clinical diagnostics, encouraged by the progressive lowering of their costs, the strong cost-effectiveness and their high sensitivity/specificity. Despite this technological advance, an important issue is represented by the validation and quality check of these tests, since they have extremely high standards of sensitivity and specificity. With appropriate controls, these assays can represent a powerful tool in the hands of laboratorists for the continuous improvement of standard Point Of Care Testing (POCT).

KEYWORDS: *Genotyping methods, Molecular diagnostics, Healthy economy, Clinical laboratory, Cost-effectiveness.*

INTRODUCTION

Upgrading of the methods for nucleic acid sequences detection is an important issue in various genetic diagnostic fields like medicine, microbiology, food and agriculture[1]. Since the genome of all organisms consist of the huge amount of DNA it is very difficult to directly detect the Single Nucleotide Polymorphisms (SNPs) or nucleotide changes in a genome. The nucleotide variation/alteration can easily be detected by amplifying specific DNA segments by several PCR-based methods[2]. Others methods[3] are developed without amplification i.e. southern blots, direct sequencing, pyrosequencing etc.

The PCR based methods are very robust for nucleic acid amplification, they are cost-effective techniques that can be performed easily in every genetic lab equipped by a dedicate instrument (i.e. thermal cycler and Real-time PCR)[4]. Although, in parallel, non-thermocycling systems Amplification were developed[5,6]. The isothermal techniques for amplification of nucleic acids including, nucleic acid sequence-based amplification (NASBA)[7], recombinase polymerase amplification (RPA)[8], helicase-dependent amplification (HDA)[9], rolling circle amplification (RCA)[10], multiple displacement amplification (MDA)[11] and loop-mediated isothermal amplification (LAMP) are alternative approaches

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that can easily be used on a constant temperature [5, 12]. In this work, we review the cost-effectiveness LAMP method and all its variants (Table 1).

LAMP

Since first time in 2000 the LAMP method and its variants have widely been used primarily for the detection of pathogens [5]. LAMP method consists of 4 sets of primers designed specifically to recognize 6 distinct sites on the target gene. The primers set including: outer primers (Fw and Rev), inner primers (FwI and RevI), and two primers, which are complementary to the downstream region of the opposite strand in the target (Fwl and Revl). The amplification products which have stem-loop DNA structures contain several inverted repeats of the target region and cauliflower-like structures with multiple loops[13,14]. The LAMP method may

TABLE 1. Reporting LAMP variants developed by several authors for several clinical field.

LAMP variants	*Clinical field (Organism)*	*Gene mutation detected*	*Sensitivity specificity of the test*	*Annotation*	*Reference*
AS- LAMP	Microbiology (Anophele Gambia)	West African-type kdr (kdr-w;L1014F)		Test in Burkina Faso population	Namountougou et al[20]
	Microbiology (Anophele Gambia)	G119S ace-1R			Badolo et al[21]
	Microbiology (Plasmodium Falciparum)	DHFR N51I	100%		Yongkiettrakul et al[22]
ARMS-LAMP	Oncology (Human)	EGFR L858R	100%	Test performed on thin layer slide in situ on Paraffin-embedded tissue. Compared with PCR-RFLP	Ikeda et al[23]
	Microbiology (Haemophila Inluenzae)	Beta-Lactamase FstI I578T	10 pg of genomica DNA	Compared with PCR-RFLP	Tamura et al[24]
	Microbiology (Fusarium Asiaticum)	F200Y mutant genotype		Compared with PCR test results	Duan et al[25]
RFLP- LAMP	Microbiology (Salmonella Shigella)	invA of Salmonella spp. and ipaH of Shigella spp			Shao et al[26]
Reverse Transcriptase RLFP-LAMP	Microbiology Differentiate Hoshino vaccine strain	Hemagglutinin neuraminidase (HN) gene digested by ScaI enzyme		Simple method to differentiate of mumps Hoshino vaccine strain from wild strains	Yoshida et al[27]
Electrochemical DNA Chip-LAMP	Metabolism (Human)	NAT2 T341C, G590A, G857A MTHFR A1298C, C677T SAA1 -13C>T		Compared with PCR-RFLP	Nakamura et al[28]
	Metabolism (Human)	CYP2D6 gene copy number	Specificity tested by NcoI and Hpy99 restriction enzymes.	Compared with PCR-RFLP	Nakamura et al[29]
Exo Proof reading-LAMP	Metabolism (Human)	ALDH2 G1951A			Kuzuhara et al[30]
PNA/LNA-LAMP	Metabolism (Human)	KRAS Codon Q12 and Q13 mutation			Itonaga et al[31]
Gold Nano-particles-LAMP	Metabolism (Human)	CYP2C19*2, CYP2C19*3 MDR1-C3435T		Compared to Pyoro-sequencing	Lu et al[33]

amplify huge amounts of DNA in a short time (around 60 min) that are usually one thousand times higher than conventional PCR. It is characterized by large reaction debris due to the production of white precipitates of magnesium pyrophosphate. This white pellet could serve as an indicator of DNA amplified or not. In the alternative, gel electrophoresis, Real-time turbidimetry, fluorescence probes, Syber Green dye can also be used for visualizing amplicons[15, 16]. In LAMP method, a large number of amplicons are produced in a short time. Since in LAMP method four sets of primer which are specific to the targeted regions are used, it has rapid amplification, a higher yield of amplification and lower detection limits in comparison to PCR based methods[17]. LAMP reaction can be performed at a constant temperature, so there is no need for thermal cycling system. Hence, it is more useful for point-of-care testing (POCT)[18]. However, the LAMP procedure has also some limitations as the perfectly melting temperature of designed primers and difficulties for using in a multiplex reaction.

LAMP based-methods for Genotyping

This method was infrequently used for the detection of the single point mutations and genotyping. Designing of new isothermal variants LAMP methods, it could a suitable alternative for genotyping technique. Moreover, the LAMP technique is very suitable, easy and cost-effective approach for POCT or on-site testing[19]. The aim of the present review is to report existing evidence for genotype detection using LAMP technique and also, discuss properties of LAMP technique.

Allele-Specific-LAMP (AS-LAMP)

AS-LAMP consists of two sets of LAMP primers able to discriminate the between wild-type to a mutant nucleotide in the target DNA sequence. Two RevI or FwI primers are usually designed as specific primers, with the mutation point at the 3'end of the Rev2 primer (5' end of the RevI primer) and an additional mismatched nucleotide to increase the specificity to each targeted nucleotide site. The other three primers, Fw3, Rev3, and FwI or RevI, are the same for the two primers sets. For each sample two LAMP reactions should be applied to each set of primers.

This method was used for the identification of the West African-type kdr in the *Anopheles gambiae* in two different studies screened for (kdrw; L1014F)[20] and G119S ace-1R mutations[21]. In another studies Yongkiettrakul et al at 2017 have also used AS-LAMP assay combined with lateral flow dipstick in order to detect N51I mutation on *DHFR* gene in *Plasmodium falciparum,* which is related to pyrimethamine resistant. The AS-LAMP method showed 100% specificity[22].

Allele Refractory Mutation Sensitive- LAMP (ARMS-LAMP)

The first ARMS- LAMP approach was performed by Ikeda et al[23] in 2007 for detection of L858R mutation of epidermal growth factor receptor (EGFR). They cast off the reaction *in situ* on thin layer slide of paraffin-embedded tissues. After deparaffinization, the LAMP reaction was performed by the FwI and RevI primers labeled with fluorescein isothiocyanate (FITC). Then, Anti FITC antibody, the biotinylated secondary antibody and HRP-conjugated streptavidin were used for the visualizing of LAMP products. The result of *in situ* LAMP was compared with PCR-RFLP results. The PCR-RFLP method could detect the mutation among 12 out of 26 among patients while *in situ* LAMP 15 patients were detected as positive for the mentioned mutation[23].

Tamura et al[24] in 2017 validated a highly resolved loop-mediated isothermal amplification method to detect the N526K ftsI mutation of β-lactamase-negative ampicillin-resistant Haemophilus influenza. They used ARMS-LAMP to accurately distinguish a different single nucleotide in the target sequence. The introduced method was capable of species-specific identification of a nucleotide (1578T) in the *ftsI* gene on Haemophilus influenzae without amplifying the point mutations (T1578G/A) in β-lactamase-negative ampicillin resistant strains. The LAMP products were visualized by measuring real-time turbidity. The detection limit of the test was 10.0 pg of genomic DNA per reaction[24].

Duan et al[25] in 2016 developed a rapid and efficient method with high specificity based on loop-mediated isothermal amplification (LAMP) for detecting the F200Y mutant genotype in benzimidazole-resistant isolates of Fusarium asiaticum. The LAMP test results were compared with PCR test results and the LAMP assay could successfully detect the F200Y mutant genotype in carbendazim-resistant isolates of *F. asiaticum* in agricultural production[25].

Restriction Fragment Length polymorphism-LAMP (RFLP LAMP)

LAMP products can also be digested with specific restriction enzymes in order to identify SNPs.

Shao et al[26] developed a multiplex loop-mediated isothermal amplification-RFLP (mLAMP-RFLP) for simultaneous detection of Salmonella strains and Shigella strains in milk. They used two sets of LAMP primers to amplify specifically target invA of Salmonella spp. and ipaH of Shigella spp[26].

Yoshida et al[27] developed a reverse transcription RT-LAMP method combined with RFLP to differentiate Hoshino vaccine strain of Mumps virus from circulating wild types. At first hemagglutinin

neuraminidase (HN) region of the virus was amplified using AMV reverse transcriptase and Bst DNA polymerase. In the HN region, the vaccine strain has a specific restriction enzyme site of ScaI. They developed a sensitive and differential method useful for laboratory surveillance for vaccine-adverse events[27].

LAMP method combined with electrochemical DNA chip

Electrochemical DNA chip (DNA chip) combined with LAMP-method was developed by Nakamura et al[28]. It is able to simultaneously identify the six polymorphisms associated with rheumatoid arthritis (RA) including N-acetyltransferase2 (*NAT2*) gene polymorphisms T341C, G590A, and G857A, methylenetetrahydrofolate reductase (*MTHFR*) gene polymorphisms C677T and A1298C, and serum amyloid A1 (*SAA1*) gene promoter polymorphism C-13T. The results were compared with the PCR-RFLP method. In the mentioned study 31 sample were genotyped and the results were completely consistent with PCR-RFLP results[28].

Using this method, Nakamura et al[28] in 2010 performed an accurate determination of *CYP2D6* gene copy number. They designed a set of primers that co-amplified the *CYP2D6* gene, but not the similar sequences of *CYP2D6*36*, and the *CYP2D8P* gene. The *CYP2D6* gene copies were determined by comparing the amount of the amplified products of the *CYP2D6* gene with *CYP2D8P* gene. For the confirmation of amplification product specificity the products were digested with NcoI and Hpy99 restriction enzymes. The amplified products were hybridized on a chip containing the probes complementary to *CYP2D6* and *CYP2D8P* genes last just for 1.5 h. The *CYP2D6* gene copies were consistent with the previous tests[29].

Exo proof reading LAMP

Exonuclease proofreading activity of 3' -5' DNA polymerase has been used in the LAMP assay developed by Kuzuhara et al[30]. In this method, a primer carrying 3' terminus nucleotide aligned at the polymorphic base of a DNA target and it was labeled with a detectable fluorescence tag on the 3' nucleotide base. If the 3' end of the primer was complementary to the target, the labeled nucleotide was continued and incorporated into the amplicon. If the primer had a mismatch with the template, the proofreading activity of the polymerase removed the labeled nucleotide and no tag was incorporated into the polymerized product. The detection of genotype was accomplished by means of fluorescence polarization without any additional cleanup. Using this method they could successfully detect G1951A SNP in the human aldehyde dehydrogenase 2 (*ALDH2*) gene[30].

Peptide nucleic acid-locked nucleic acid (PNA-LNA) mediated LAMP

PNA-LNA mediated LAMP method is an elegant and highly specific method designed by Itogana et al[31] at 2016 for the detection of low mutant *KRAS* Q12 and Q13 in a large excess of wild-type DNA. They used PNA-LNA mediated LAMP for mutation detection. In PNA-LNA mediated LAMP, a clamping PNA probe specific for the wild-type nucleotide and additional LNA primers complementary to the mutant type nucleotide were designed for the looped region of the primary LAMP products. The LAMP reactions were conducted at an isothermal condition of 65°C using a strand displacement DNA polymerase. In that method the FwI and Rev3 primers anneal and extend on the target DNA and the newly synthesized DNA chains are displaced by extension of Fw3 and Rev3 respectively. In wild-type samples the PNA probe generates stem -loop structures with displaced products that prevent annealing to the matching of the LNA primer. When the target sequence is mutated, the PNA cannot form a clamp with displaced DNA because of a single-base mismatch and as a result the LNA primer binds and anneals to the target region and consequently the extending is continued. For the detection of amplified product Real-time PCR equipment, agarose gel electrophoresis and the naked-eye system were applied. The *KRAS* point mutation by PNA-LNA mediated LAMP was also compared with direct sequencing assay and PNA clamping PCR. Mutant alleles could be reproducibly detected with a mutant-to-wild type ratio of 30% by direct sequencing and of 1% by PNA-clamping PCR, while the mutant alleles could be detected within 50 min in samples diluted down to a mutant-to-wild type ratio of 0.1% using LAMP method[32].

Invasive reaction coupled with oligonucleotide probe-modified gold nanoparticles

Lu et al[33] in 2017 introduced new technique derived from the LAMP method for SNP detection. First multiple fragments containing the SNPs of interest are amplified using a set of specific primers. One for normal allele and another for mutated allele. Next, a subsequent invasive reaction using Afu flap endonuclease (FEN) is performed. In the presences of the targeted allele, the wild-type allele forms an overlapping structure which is recognized and cleaved by Afu enzyme.

Since the allele-specific probe is sensitive to discriminate single base, they claimed that it is possible to use a multiplexed LAMP to generate amplicon mixtures for multiple SNP detections.

The authors designed a Multiplex-LAMP protocol for Genotyping of 3 SNPs *CYP2C19*2*, *CYP2C19*3* and *MDR1*-C3435T useful in the phar-

macogenomic of clopidogrel. The method is enough sensible to give an accurate genotyping result by starting 100 copies of genomics. The method retried identical typing results when compared with the results of Pyrosequencing method[33].

CONCLUSIONS

In recent years different methods have been developed for genotyping of SNPs and mutations in nucleic acid sequences. These methods have several applications ranging from agriculture to medicine[17]. The LAMP method for the first time was introduced by Notomi et al[5] at 2000 and since then different methods derived from LAMP have widely been used for the detection of pathogens.

LAMP method can be easily performed in any laboratory with basic isothermal equipment. It is very cost-effective and can easily be adapted to detect hot spot mutations in all genes[34]. Our review confirms that it is possible to reduce the time of detection in any kind and in any place of the samples must be analyzed in order to identify the nucleotide replaced. In the next future the physicians will have new means based on the individual genetic information, to plan the personal treatment for their patients in order to maximize benefits and minimize toxicity[35, 36]. Therefore, it is fundamental that pharmaceutical and biotechnology companies join, in order to develop an extensive study on the standardization method to validated tests suitable for routine diagnostics in genomics[37].

The integration of genotype testing in the routine clinical practice is an ongoing process. It appears obvious that the usefulness of these results from the LAMP methods in the clinical practice severely depends on the validation of this diagnostic tools, as well as on specialized genomic expertise to efficiently interpret the results of the tests[38].

Conflict of Interest:

The Authors declare that they have no conflict of interests.

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