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Advances in Loop-Mediated Isothermal Amplification (LAMP) Technology and Its Necessity to Detect Helminth Infections: An Overview

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Introduction

Use of nucleic acid (NA) markers in clinical diagnosis of infections is rapidly making its way in the clinics and has vast potentialities of improvement of medical facilities for the common man. The traditional methods of diagnosis often leads to erroneous results since the clinical signs of many infections are mostly non-specific and may have multiple sources of origin [1]. Proper treatment depends on proper diagnosis of the disease. The lack of accuracy in the diagnostic methods cost human lives and high rates of morbidity in endemic populations around the world [2]. The situation becomes more serious in case of helminth infections because the identification of helminths has its own complexities. The helminths are usually identified from the eggs or any body part (like the segments of proglottids in cestodes) extruded along with the faeces of the definitive host. However, the eggs and/or the body parts in many cases appear to be similar and create confusions in their proper identification. The eggs of *Taenia* spp., for example, appear very similar and identification beyond the generic level becomes almost impossible leading to doubtful results [3]. Traditional parasitological diagnostic techniques like Kato-Katz assay for counting eggs in faeces, though inexpensive, lack specificity and sensitivity [4]. An added disadvantage is that the morpho-anatomical characteristics of the helminths reportedly vary with age, development and physiological modifications that augment the confusions [5]. In many cases, as in Diphyllobothrium infections, the entire worm is not available for identification [6]. The intermediate stages of the helminths are also not easily available. They remain encysted in the internal organs of the intermediate hosts and cannot be obtained for antemortem diagnosis [2]. The serology-based diagnostic techniques also come with disadvantages like cross-reactivity and false-positive results [7]. Molecular diagnostic tools provide an effective alternative to these traditional methods and are much higher in specificity and sensitivity [8]. Parasitic DNA serves as molecular biomarkers and has been exploited by the modern technologies to detect their presence in the human host [9]. Human clinical samples like sera [10], plasma [11], urine [12] and faeces [13] have been used to detect the presence of parasitic DNA. However, most of these techniques heavily use the Polymerase Chain Reaction (PCR) that is extremely expensive and can rarely be afforded by the lower income group countries. Therefore, development of cost-effective, rapid and sensitive technique is a necessity to provide the benefits of molecular technologies to the common man. Loop mediated isothermal amplification technique (LAMP) seems to be a hope to make molecular technologies feasible to everyone.

The current paper presents a short review on the development of many aspects of LAMP since its inception to the current integration of LAMP on lab-on-a-chip (LOC) device. It also briefly discusses its applicability in various fields. Finally its importance in detection of helminth infections have been analysed considering the dearth of research in the field.

Loop Mediated Isothermal Amplification (Lamp) Technique and Its Developments

The LAMP technique initially developed by Notomi et al. [14] employed a DNA polymerase and a set of four primers (two inner and two outer primers) that recognised six distinct sequences of the target DNA. The DNA sample (containing the target sequence) and the four primers were at first denatured by heat followed by rapid cooling on ice [14]. The LAMP reaction was then initiated by adding Bst DNA polymerase large fragment [It is the portion of *Bacillus stearothermophilus* DNA polymerase protein containing the 5'→3' polymerase activity but lacking the 5'→3' exonuclease activity] and the process was carried out for 1 h. at 65°C [14]. The reaction began with the binding of of the inner primers containing sequences of the sense and anti-sense strands of the target DNA [14]. This was followed by strand displacement DNA synthesis by the outer primers. Subsequently, the cyclical amplification and elongation and recycling lead to the amplification of the target sequence [14]. LAMP can also be done on a RNA target using reverse transcriptase and Bst polymerase [14].

Perhaps the best part of LAMP is that it can easily be modified and the modifications done so far have only made it more and more handy and simple. This has given a direction for LAMP to be developed for use in resource-limited settings following the ASSURED guidelines of World Health Organisation (WHO) {"Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment free, Deliverable to end users" [1]}.

Simplification in isolation of the DNA

Considerable works have been done to reduce the elaborate and multi-step procedure of DNA isolation and its purification. Qiao et al. [15] extracted DNA from *Bacillus anthracis* by boiling the bacteria at 95°C to 100°C for about 30 min in sterile water. They found that it was sufficient for the LAMP reaction. Hatano et al. [16] however applied the phenol and chloroform method for the extraction of DNA from *Bacillus anthracis*. Grab et al. [17] added detergents to the clinical samples like blood and cerebrospinal fluid to increase the sensitivity of detection of trypanosomiasis infection by the LAMP method. Mikita et al. [18] developed the direct boil-LAMP method for the detection of cutaneous leishmaniasis infections. Sriworarat et al. [19] also utilized the direct boil-LAMP method for detection of Leishmania infection in which clinical samples like blood, saliva or tissue biopsies (collected from patients with cutaneous and visceral leishmaniasis) were boiled for 10 min and directly introduced into the LAMP reaction mixture omitting the purification of DNA. The method followed by Mikita et al. [18] and Sriworarat et al. [19], using crude DNA, did not affect the result. The method was proposed to be used in mass screening programmes in endemic regions with low resource settings [18, 19]. Dhumpa et al. [20] demonstrated that magnetic beads could efficiently extract DNA from biological samples with high selectivity and sensitivity. Sun et al. [21] went a step further and investigated whether the magnetic beads used for DNA extraction in any way had inhibitory effects on the LAMP reaction. In order to test this they carried out the LAMP amplification in the presence of magnetic beads at various concentrations and found that the beads did not have any inhibitory effect on LAMP reaction. The use of magnetic beads for DNA extraction had many other advantages as well. Sun et al. [21] devised a highly mobile single chambered lab-on-a-chip (LOC) system where the magnetic bead – DNA complex could be washed and the LAMP reaction mixture could be directly added for amplification and detection of the target gene. Earlier attempts to develop such LOC systems employing LAMP reactions were found to be complicated [22] and utilised two separate chambers for DNA purification and DNA amplification [23]. This not only increased the size of the set-up due to additional pumps and fluid transfer system but also added to its cost [21]. All these limitations on the practical applicability of the LAMP-based LOC system were overcome by the single chambered chip of Sun et al. [21].

Detection of the LAMP products

The detection of the LAMP products by electrophoreses and blotting techniques used by Notomi et al. [14] has come a long way to its current visual detection techniques. Mori et al. [24] utilized the turbidity developed in the LAMP reaction mixture by a white precipitate of magnesium pyrophosphate as the indicator of a positive LAMP reaction. Absence of turbidity indicated a negative LAMP reaction [24]. The pyrophosphate was obtained as a by-product in the LAMP amplification process and the formation of an insoluble salt by combination of the pyrophosphate with a divalent metallic ion like magnesium ion (Mg++) was the principle underlying the development of turbidity [24]. As the increase in turbidity of the reaction mixture was correlated with the formation of the pyrophosphate precipitate which in turn depended upon the amount of DNA synthesized, real time monitoring of the LAMP reaction could easily be attained by measurement of turbidity using a real-time turbidimeter [25]. However, it was later observed that addition of the extra divalent metal ion for increasing the amount of precipitate could affect the specificity of the reaction [26]. Tomita et al. [27] suggested the use of calcein, a fluorescent metal indicator, added in the reaction mixture could enhance the visual detection of LAMP reaction by the alteration of fluorescence intensity (measured by UV irradiation) during the course of reaction. Other fluorescence dye of similar nature is hydroxyl naphthol blue [28]. Intercalating dyes like ethidium bromide [29] and PicoGreen [30] have also been used as detection agents for the LAMP amplification products. Malachite Green has been successfully used in colorimetric LAMP approaches for detection of *Leishmania* spp. [31] and *Plasmodium* spp. [32]. Use of fluorescent dyes like SYBR Green [33] that needs to be added to the reaction mixture after the reaction has its own shortcomings as it may lead to contamination when the tubes are opened to add the dye [28]. The fluorescent dyes are limited in their application because they can bind non-specifically even to the primers leading to erroneous interpretation of results [28]. The specificity of the LAMP product detection could be increased by addition of fluorescent molecular beacon probe targeted to an internal sequence of the amplicons [34]. Use of lateral flow dipstick format devised by Njiru [35] could also help in minimizing non-specific signals. The inhibitory effects of several fluorescence dyes on LAMP reaction was assessed by Sun et al. [21]. The experiments performed by Sun et al. [21] showed that SYTO-24, SYBR Safe, SYBR Green and Eva Green were not suitable to be used in real time LAMP. SYTO-82 could be used as an indicator but it had disadvantages of high background noise [21]. Sun et al. [21] strongly advocated the use of SYTO-62 as the fluorescent indicator in real time LAMP.

The methods that depend on visual detection of LAMP amplification products utilises optical-electrical devices for getting the result outputs that are bulky and expensive [36]. This handicaps the production of a gene chip [36]. The detection of DNA by electrochemical methods has been proposed to be a better alternative in this regard [37]. The cyclic voltammetry method has been found to have high specificity for DNA detection using a gene chip [36]. It has also been proposed to be faster and cheaper allowing measurements under varied experimental conditions [38]. Considering all these factors Jiang et al. [36] strongly advocated the use of cyclic voltammetry for the electrochemical detection of DNA. The principle of this technique lies in the fact that combination of the DNA amplification products with a positive dye like crystal violet or methylene blue reduces the oxidation peak current (ipA) and reduction peak current (ipC), these changes of ipA/ipC are proportional to the amounts of DNA amplification products and can be measured by an electrode helping in the

quantitative detection of DNA [36]. Cyclic voltammetry method was reported to have high specificity, sensitivity and accuracy. It was found to have good anti-interference ability against Aspirin and Vitamin C within the sample [36]. All these features enable electrochemical detection of DNA to be used in gene-chips or other such small-sized portable devices for field studies [36].

Power source for LAMP

The power source for operation of the LAMP set up under field conditions was the most essential and challenging part of its development because it heavily relied on use of electricity to run the test. This posed many difficulties for its operation in field conditions and in places with poor/no electricity supply. Hatano et al. [16] came up with pocket-warmer LAMP in a Styrofoam box which was highly mobile and sensitive. The pocket-warmer act as a source of heat for LAMP and therefore does not depend on electricity [16]. This property enabled it to be used for field studies [16]. Though the device was developed for anthrax detection (*Bacillus anthracis*) it may be used for detection of infectious diseases in areas lacking proper electricity infrastructures [16]. Dominguez et al. [34] used a PortaTherm phase-change incubator for maintenance of a stable, constant temperature of 37°C for an interference gamma release assay. The PortaTherm was based on using substances that change their phase from solid to liquid (and vice versa) at a constant phase-change temperature that is maintained until the phase change has been completed [34]. Dominguez et al. [34] utilized a substance (the substance was not specified in their paper) with a phase-change temperature of 37°C that was packed in plastic bags, heated in a water bath and cooled till crystals had just started to form when the plastic bags were placed in an insulated chamber like a Styrofoam box. This constituted the PortaTherm and could maintain a stable temperature of 37°C for about four days if it was not opened [34]. This low-cost, portable, insulated incubator was a good alternative to electricity and could be used in field studies [34]. LaBarre et al. [1] utilized an engineered exothermal chemical heating unit for maintenance of constant temperatures that is an essential pre-requisite for isothermal nucleic acid amplification. The engineered exothermal chemical heating unit consisted of calcium oxide (CaO) and an engineered phase change material (EPCM) (not specified in the paper) [1]. The principle of heat generation lied in the fact that after the heat from the CaO reaction begins to melt the EPCM, the extra heat generated in the exothermic reaction is converted into the latent heat of fusion of the EPCM [1]. When the CaO reaction reaches equilibrium the latent heat (acting as an energy source) helps in keeping the two-phase EPCM at the desired temperature until the solidification has been completed [1]. Other experiments performed showed that use of only CaO as the heat-source when compared with CaO-EPCM interface, the later was found to be more effective in proper temperature control [1]. An added advantage of the prototype was that highly pure CaO need not be used which cut the costs greatly and made it much cheaper [1]. LaBarre et al. [1] also highlighted the fact that the LAMP reagents used could be dried in the reaction-tubes that could help omit using a freezer for transport and storage thereby eliminating the use of yet another energy-source. The ESE Quant Tube Scanner has been proposed to be a very suitable and portable device using rechargeable batteries as the energy source [28]. Other advantages of the device were that the products could be detected after a single-step amplification using software installed in a laptop and was small and light in weight [28]. Njiru [28] also proposed the use of rechargeable solar batteries as alternative energy-source. According to Njiru [28] sodium acetate was non-toxic and could easily be recharged by simple boiling in water allowing it to be reused more than once, therefore sodium acetate heat pads when packed in proper insulated units could serve as a good energy source for LAMP reactions. Sun et al. [21] utilised rechargeable batteries as energy source in their experiments.

Primers and their design

The conventional LAMP technique developed by Notomi et al. [14] utilised four primers (two inner and two outer) and a DNA polymerase with strand displacement activity. A more rapid form of the technique was later developed by Nagamine et al. [39]. The technique was the same as conventional LAMP with additional loop primers that could bind to additional sites (stem-loops) that could not be accessed by the inner primers [39]. Application of the loop primers could reduce the LAMP reaction time to almost half of the conventional LAMP technique [39]. This rapid form of LAMP found various clinical applications like rapid detection of periodontopathic bacteria (*Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola*) by Yoshida et al. [40] and rapid detection of *Enterococcus faecalis* in infected root canals by Kato et al. [41].

The design of the primers is also very simple. They can easily be designed online by the primer design support software: http://primerexplorer.jp/e/ [42].

Applications of LAMP

Since its advent, LAMP has found applications in various fields. LAMP has been successfully used to determine sex of pre-implantation bovine embryos that can be used for the development of animal husbandry [43]. A similar experiment performed by Khamlor et al. [44] also used LAMP in determining sex of bovine embryos before embryo transfer and opined that it could help in increasing the number of animals of the desired sex and boost the animal industry. Use of LAMP to fight bioterrorism (like the anthrax bacteria, *Bacillus anthracis*) has been highlighted by Hatano et al. [16]. Iwamoto et al. [45] utilised LAMP for the rapid detection of *Mycobaterium tuberculosis* complex from clinical sputum samples. Kouzaki et al. [46] utilised LAMP reaction to differentiate between Bacillus Calmette-Guérin (BCG) *Mycobacterium bovis* strain from other *Mycobaterium tuberculosis* complexes. BCG is used as attenuated vaccine against *Mycobacterium tuberculosis* and as a prophylaxis against bladder cancer [46]. It causes infectious diseases in immuno-suppressed patients which necessitates the

differentiation between BCG *M. bovis* strain and other *Mycobaterium tuberculosis* complexes [46].

The reverse transcriptase LAMP method has also been devised that have been used for the detection of subtype of H7N9 avian influenza virus [47]. The method could detect the presence of H7N9 avian influenza virus RNA in clinical samples of chickens, pigeons, human, drinking water, soil and cloacal/tracheal swabs [47]. The reverse transcriptase LAMP also finds application in detection of metastasis of gastric cancer [48] and other similar oncological cases [49].

LAMP has found important applications even in determination of pathogens in food [50] and detection of genetically modified ingredients in domestic and imported foods [51].

The LAMP technology has also been used for detection of various parasitic infections like malaria [52, 53] and leishmaniasis [19]. Fernández-Soto et al. [9] applied the technique to detect *Schistosoma mansoni* infections from faecal and sera samples of infected mice. LAMP has been employed for the differentiation of different species of Taenia (*T. solium, T. saginata, T. asiatica*) from eggs in stool samples [54] and detection of *Necator americanus* infections from human stool samples [8]. Song et al. [55] successfully used a microfluidic chip LAMP (LOC-LAMP) device to detect the presence of *Schistosoma mansoni* in an infected mice host. An important point needs to be mentioned here is that there is dearth of works related to application of LAMP in the detection of helminth infection though helminths are one of the major causes of morbidity in the developing parts of the world [2]. Apart from this, there are various complexities involved in identification of helminths [2-4], which makes molecular technologies more reliable, sensitive and specific for proper antemortem diagnosis of helminth infection. The routine clinical diagnostic techniques used for helminth infections are also not reliable leading to wrong treatments and survival of the helminths in unsuspecting populations [8]. The LAMP integrated on a gene chip (like the one used by Song et al. [55]) can also be used in field assays of meat samples (like beef and pork) to screen for the presence of developmental stages of the helminths (like the cysticercus stages) and act as a form of food security. Proper researches in this field are still lacking and are necessary to combat the helminth burden throughout the world.

Conclusion

The development of molecular technologies was a boon for the humans because it could be used to detect and diagnose various infections easily and efficiently. Making the diagnostic techniques patient-centric was the main aim behind the advent of point-of-care-test kits [22]. The main requirements for such kits/devices were small size, light weight, easy to operate and reusability following the ASSURED guidelines laid down by the World Health Organisation (WHO) [1]. LAMP has many advantages: (i) it can amplify DNA without being affected by the presence of non-target DNA enabling crude DNA to be used in such studies, (ii) DNA amplification takes place at isothermal conditions making the process independent of the use of the expensive thermal cyclers, simple heating devices could fulfil the temperature requirement, (iii) the detection of the LAMP amplification products is very simple (turbidity, fluorescence or electrochemical detection) and not necessarily depend on elaborate processes like electrophoresis, (iv) isolation of DNA for LAMP could be simplified like direct boiling in sterile water or use of magnetic beads, and last but not the least (v) the process is also applicable to RNA by using reverse transcriptase enzymes. All these advantages together with the efforts of workers around the world could help it develop into microfluidic system based lab-on-a-chip device. The use of cyclic voltammetry for the quantitative detection of DNA by electrochemical methods [36] helps to do away with the bulky and heavy optical devices to see the result. This was indeed an important step towards the development of small sized lab-on-a-chip device. The contribution of Sun et al. [21] in this field towards the development of a single-chambered simplified LOC system for DNA purification and amplification is also praise-worthy. Efforts of researchers like Hatano et al. [16] and LaBarre et al. [1] towards development of alternative energy devices for LAMP to make it independent of electricity also cannot be neglected. Loopamp Malaria Pan/Pf detection kits used by Cook et al. [53] and Sema et al. [56] for mass diagnosis of malaria was a major step towards non-instrumented nucleic acid amplification by LAMP (NINA-LAMP). NINA-LAMP utilise electricity-free heaters. However, as already mentioned, the development of such kits for diagnosis of helminth infections is also necessary considering lack of enough research in the field. Such kits will be extremely useful for mass screening of helminth infections in endemic populations and taking suitable control measures. They can also be used to test water, soil, plant and animal products for the presence of developmental stages (eggs/larvae) of the helminths and prevent its transmission. It will not only be important to humans but also to livestock and zoo-animals that are important reservoirs of helminthes around the world.

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