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REVIEW ARTICLE

Loop-mediated isothermal amplification (LAMP): a versatile technique for detection of micro-organisms

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Summary

Loop-mediated isothermal amplification (LAMP) amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions by using a DNA polymerase with high displacement strand activity and a set of specifically designed primers to amplify targeted DNA strands. Following its first discovery by Notomi *et al.* (2000 *Nucleic Acids Res* **28**: E63), LAMP was further developed over the years which involved the combination of this technique with other molecular approaches, such as reverse transcription and multiplex amplification for the detection of infectious diseases caused by micro-organisms in humans, livestock and plants. In this review, available types of LAMP techniques will be discussed together with their applications in detection of various micro-organisms. Up to date, there are varieties of LAMP detection methods available including colorimetric and fluorescent detection, real-time monitoring using turbidity metre and detection using lateral flow device which will also be highlighted in this review. Apart from that, commercialization of LAMP technique had also been reported such as lyophilized form of LAMP reagents kit and LAMP primer sets for detection of pathogenic micro-organisms. On top of that, advantages and limitations of this molecular detection method are also described together with its future potential as a diagnostic method for infectious disease.

Introduction

Nucleic acid amplification is one of the most valued methods across all fields of science, from diagnosis of infectious diseases to application in biotechnology (Parida *et al.* 2008). In addition to the widely used polymerase chain reaction (PCR)-based detection, numerous amplification techniques were designed (Guatelli *et al.* 1990). Examples of popular methods used are nucleic acid sequence-based amplification (Malek *et al.* 1994), self-sustained sequence replication (3SR; Fahy *et al.* 1991) and strand displacement amplification (SDA; Walker *et al.* 1992). Each of these methods has a different set of requirements to initiate DNA synthesis. For instance, PCR involves heat to denature the double-stranded target DNA into single-stranded to initiate DNA synthesis. SDA uses strand displacement action of exo-klenow to produce target DNA copies from 5′ to 3′ and the procedure occurs at a single temperature (Walker *et al.* 1992). These methods are widely used in all fields of science to amplify the target sequence. However, they still suffer from insufficiencies and inadequacies. All these methods require a sophisticated instrument for amplification and detection or elaborate methods for performance (Fang *et al.* 2008). As a result, researchers and scientists have worked hard to overcome these limitations.

In the past decade, numerous isothermal amplification techniques were established. These include recombinase polymerase amplification, rolling circle amplification and helicase-dependent amplification (Zanoli and Spoto 2013). Finally, Notomi *et al.* (2000) successfully devised a novel isothermal amplification method to amplify a limited amount of DNA copies into a million copies within an hour. This method is known as loop-mediated isothermal amplification (LAMP).

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LAMP is a novel molecular technique of nucleic acid amplification where a set of four (or six) different primers binds to six (or eight) different regions on the target gene making it highly specific (Notomi *et al.* 2000). This primer set consists of two outer (F3 and B3) primers, two inner primers (forward inner primer (FIP) and backward inner primer (BIP)) and loop primers (loop forward and loop backward). LAMP reaction can be done simply at an isothermal condition (Chen *et al.* 2015) by using *Bst* DNA polymerase which have high-displacement activity (Notomi *et al.* 2000). An illustration on the LAMP primers binding sites and the amplification process is given in Fig. 1.

Different forms of LAMP assays

Since its development in Notomi *et al.* (2000), LAMP has undergone numerous advancement in terms of its applications as a molecular amplification technique. In this section, different forms of LAMP assays including conventional LAMP, reverse-transcription LAMP, multiplex LAMP and a few other LAMP forms for detection of micro-organisms will be discussed in details.

Conventional LAMP

LAMP was successfully demonstrated to detect human respiratory pathogens, such as *Mycobacterium tuberculosis* (Kumar *et al.* 2014; Bentaleb *et al.* 2016; Nagai *et al.* 2016; Sethi *et al.* 2016; Sharma *et al.* 2016; Kaewphinit *et al.* 2017), *Streptococcus pneumoniae* (Seki *et al.* 2005; Xia *et al.* 2014), *Bordetella pertussis* (Fujino *et al.* 2015; Brotons *et al.* 2016; Kamachi *et al.* 2017), and carbapenem-resistant *Klebsiella pneumoniae* (Nakano *et al.* 2015). Furthermore, LAMP was also devised to diagnose pathogens associated with food-borne diseases, for

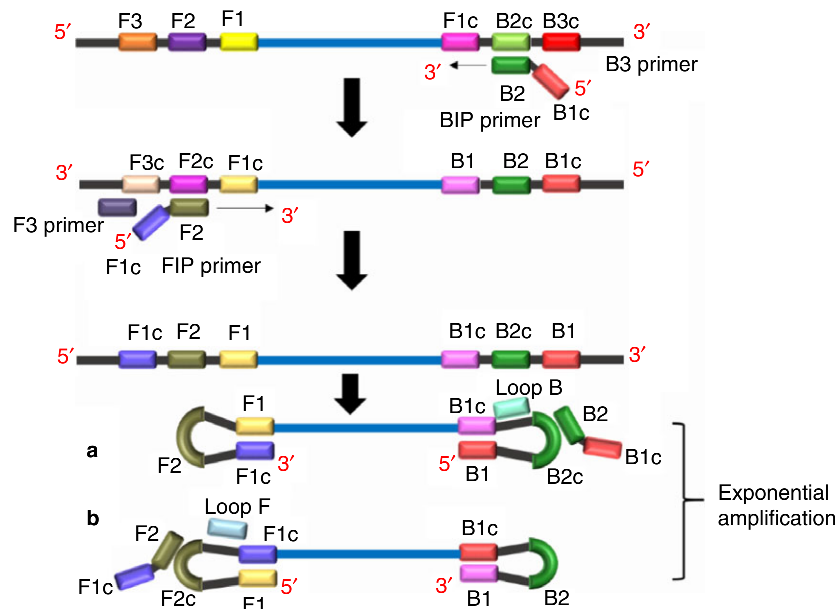

Figure 1 Schematic representation of LAMP reaction and its principle. LAMP reaction involved reaction mixture consists of dNTPs mix, *Bst* polymerase, fluorescence dye, primers and DNA template. Primer design: Design of primer for LAMP reaction and is characterized by the use of four different primers specially designed to recognize six distinct regions of the target DNA. Forward Inner Primer (FIP) consists of a F2 region at the 3′-end and an F1c region at the 5′-end; Forward Outer Primer (F3 Primer) consists of a F3 region which is complementary to the F3c region of the template sequence; Backward Inner Primer (BIP) consists of a B2 region at the 3′-end and a B1c region at the 5′-end. Backward Outer Primer (B3 Primer) consists of a B3 region which is complementary to the B3c region of the template sequence. LAMP reaction: The amplification starts when F2 region of FIP hybridizes to F2c region of the target DNA and initiates complementary strand synthesis, follow by F3 primer that hybridizes to the F3c region of the target DNA and extends, displacing the FIP linked complementary strand. This displaced strand forms a loop at the 5′-end. This single-stranded DNA with a loop at the 5′-end then serves as a template for BIP. B2 hybridizes to B2c region of the template DNA. DNA synthesis is initiated leading to the formation of a complementary strand and opening of the 5′-end loop. Subsequently, B3 hybridizes to B3c region of the target DNA and extends, displacing the BIP linked complementary strand. This results in the formation of a dumbbell-shaped DNA. The nucleotides are added to the 3′-end of F1 by *Bst* DNA polymerase, which extends and opens up the loop at the 5′-end. The dumbbell-shaped DNA now gets converted to a stem-loop structure (refer a and b). This structure serves as an initiator for LAMP cycling, which is the second stage of the LAMP reaction. Loop primers can be added as well for exponential amplification of LAMP. The final products obtained are a mixture of stem-loop DNA with various stem lengths and various cauliflower-like structures with multiple loops. Fig. 1 was modified from Notomi *et al.* (2000). [Colour figure can be viewed at wileyonlinelibrary.com]

instance, *Salmonella typhi* (Abdullah *et al.* 2014; Fan *et al.* 2015), *Campylobacter jejuni* and *Campylobacter coli* (Pham *et al.* 2015), and *Helicobacter pylori* (Bakhtiari *et al.* 2016; Yari *et al.* 2016). Other successful examples of LAMP usage include *Neisseria meningitidis* (Lee *et al.* 2015), *Listeria monocytogenes* (Birmpa *et al.* 2015; Wang *et al.* 2015b; Ye *et al.* 2015), *Entamoeba histolytica* (Rivera and Ong 2013; Singh *et al.* 2013; Mwendwa *et al.* 2017), and human leptospirosis (Seesom *et al.* 2015; Hsu *et al.* 2017). LAMP was also efficiently formulated against several multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Kim *et al.* 2016b). The high LAMP assay usage proves its potential as a means for accurate molecular detection and to differentiate human pathogens.

Recently, LAMP was used for the detection of a wide range of viruses. For example, adenovirus (Ziros *et al.* 2015), varicella zoster (Kobayashi *et al.* 2014; Niihara *et al.* 2017) and cytomegalovirus (Wang *et al.* 2015a). Hepatoviruses such as hepatitis B (Zhao *et al.* 2016) can also be detected using LAMP technique. The results showed that LAMP assay is promising for hepatitis diagnostics, donor screening, epidemiological studies and therapeutic monitoring of patients undergoing antiviral treatment.

Protozoan parasites are equally harmful to humans and are one of the leading causes of death in the world. A parasitic disease detected by using LAMP was for malaria diagnosis of *Plasmodium* spp. (Britton *et al.* 2016; Lau *et al.* 2016; Zhang *et al.* 2017). Poole *et al.* (2015) developed a highly sensitive LAMP for *Loa loa* detection, targeting the *RF4* genes. The study reported that *RF4*-specific LAMP can detect 1 microfilaria which is equivalent to 100 pg within 30 min. These studies provide examples of how LAMP can be implemented in field detection and early management of the parasitic infection (Poole *et al.* 2015).

A research was conducted by Nzelu *et al.* (2014) to detect *Leishmania*. In the study, they developed LAMP and tested it with 122 suspected clinical samples. The study showed that LAMP was efficient to detect 10^1 copies of DNA. They concluded that the newly developed LAMP was helpful when leishmania parasite density is very low, especially during the early infection stage (Nzelu *et al.* 2014, 2016). There was a number of other reported significant practices of LAMP including *Trichomonas viganalis* (Reyes *et al.* 2014), *Necator americanus* (Mugambi *et al.* 2015) and *Strongyloides stercoralis* (Watts *et al.* 2014), and it was shown that LAMP is at least 1000 times more sensitive than the conventional PCR.

The routine detection of fungi is laborious and expensive. A recent study was carried out to detect an extensive range of fungi, including *Candida albicans*, *Cryptococcus neoformans* and *Mucor racemosus*. The study reported the sensitivity of LAMP assay was 10^2 copies per 25 μl reaction. The assay can be efficiently used to detect fungi from the environment (Nakayama *et al.* 2017). Another study was carried out by Luo *et al.* (2014) to detect aflatoxins by *Aspergillus flavus, Aspergillus parasiticus* and *Aspergillus nomius*. The study developed real-time LAMP to quantify *A. flavus*, *A. parasiticus* and *A. nomius,* and stated the detection limits of 10^1, 10^2 and 10^3, respectively, at 65°C within 60 min. The real-time LAMP assay established could be a promising assay for early diagnosis of *Aspergillus* infection in humans (Luo *et al.* 2014).

Other successful examples of LAMP usage to detect fungal infections include *Fonsecaea* species (Sun *et al.* 2010), *Trichosporon asahii* and *Pneumocystis jirovecii* (Nakashima *et al.* 2014; Singh *et al.* 2015). LAMP requires minute quantities of DNA for detection of the fungal pathogen with unrivalled high sensitivity and easy handling. These qualities make LAMP suitable for all ranges of applications in the diagnosis of communicable diseases.

Reverse transcription LAMP

Reverse transcription loop-mediated isothermal amplification (RT-LAMP) is a one-step nucleic acid amplification technique used by taking a step further and adding LAMP with a reverse transcriptase enzyme to allow RNA detection (Notomi *et al.* 2000). Like RT-PCR, RT-LAMP uses reverse transcriptase to make complementary DNA from RNA and is further amplified by using DNA polymerase. This method is valuable and beneficial because the assay can be completed in a single step, by incubating all the primers and enzymes (Bst polymerase and reverse transcriptase) with a constant temperature (Notomi *et al.* 2000, 2015; Mori *et al.* 2013), and this was very effective in detecting viruses with an RNA genome.

RT-LAMP was established for Dengue virus (Hu *et al.* 2015; Lau *et al.* 2015), influenza viruses (Nakauchi *et al.* 2014; Bao *et al.* 2015; Kim *et al.* 2016a), Hepatitis C (Nyan and Swinson 2016; Zhao *et al.* 2017), Ebola virus (Oloniniyi *et al.* 2017), respiratory syncytial virus (Hoos *et al.* 2017), zika virus (Tian *et al.* 2016; Wang *et al.* 2016; Chotiwan *et al.* 2017) and Middle East Respiratory Syndrome coronavirus (Mers-CoV) (Li *et al.* 2015; Lee *et al.* 2016). Apart from helping in diagnosis, the RT-LAMP can also be used as an epidemiologic surveillance system for human virus infections.

Currently, one of the most successful uses of RT-LAMP is diagnosing the HIV retrovirus. RT-LAMP was developed to diagnose dormant HIV stage in infected individuals (Ocwieja *et al.* 2015; Odari *et al.* 2015). A

recent advancement was carried out by Damhorst *et al.* (2015) by incorporating a digital device with RT-LAMP to provide a smartphone-image of positive HIV-1 detection from whole blood. The assay showed sensitivity of 670 viruses per microlitre of whole blood. The digital RT-LAMP method could be used for the detection of viral load from a simple finger prick of blood from HIV-positive individuals in primary care amenities and clinical laboratory. The assay is functional for hospital-based infection control and for laboratory-based observations (Damhorst *et al.* 2015).

A recent study by Tian *et al.* (2016) also used RT-LAMP for the detection of synthetic zika virus (ZIKV) by targeting the NS5 gene. Although the study was only tested with synthetic ZIKV, it was one of a few studies that used a dual enzyme activity, *Bst* 3.0 polymerase which could amplify both DNA and RNA under the same conditions. The sensitivity of the assay was reported to be 1 attomolar (aM) synthetic ZIKV within 30 min. The study also reported no cross-reactivity with closely related Flaviviruses (Tian *et al.* 2016).

RT-LAMP was also used to detect plant pathogens. One of the recent examples of RT-LAMP plant pathogens detection is Apple chlorotic leaf spot virus (ACLSV), by targeting the virus coat protein gene. The reaction conditions were optimized according to temperature and reaction time. The RT-LAMP developed was capable to detect concentration as low as 0·02 μg μl^{-1} at 64°C. The study reported that RT-LAMP has a 100-fold greater sensitivity as compared with RT-PCR (2·29 μg μl^{-1}). The assay could be successfully applied to field-collected apples to diagnose the diseases and to potentially control the spreading of ACLSV (Peng *et al.* 2017).

RT-LAMP was as well developed for animal pathogens, for example, Batai virus (BATV), a mosquito-borne virus, which would cause congenital defects in ruminants (cattle). Liu *et al.* (2016) developed and optimized RT-LAMP targeting the *M* gene of BATV. The time detection of BATV was reduced to 40 min, at 65°C, when RT-PCR would normally take hours. The results were simply and readily visualized with the naked eye on colour change. The assay reported a sensitivity of 2·86 copies per μl, which is roughly 100 folds higher than qRT-PCR. Therefore, RT-LAMP showed a promising diagnostic tool for BATV detection, especially in the farm house (Liu *et al.* 2016).

A novel RT-LAMP was developed by Chander *et al.* (2014) and it was the first study to report the application of novel polymerase, OmniAmp polymerase. The polymerase enzyme exhibited similar activity to that of *Bst* 3.0 polymerase that allows the direct detection of both DNA and RNA. RT-LAMP assays were developed for six RNA viruses. All targets were amplified within 30 min without extra steps or alteration of the reaction designs used for DNA targets. In comparison to the conventional *Bst* polymerase, OmniAmp polymerase was reported to be less inhibited by whole blood components, more rapid detection for diluted low viremia samples, more thermostable and perfect with a dry set-up that allows extended storage period at ambient temperature, thus it was more suitable for the development of lateral flow device and lyophilized LAMP. Considering the qualities, OmniAmp polymerase is more suitable to be used for molecular diagnostic in field setting (Chander *et al.* 2014).

Multiplex LAMP

Simultaneous detection of multiple target genes could enhance the specificity and reliability of a diagnosis method. This was applied in LAMP reaction as an advancement of the method, namely multiplex LAMP (mLAMP). Lau *et al.* (2015) developed RT-LAMP in a single tube coupled with mLAMP approach for detection of the dengue virus. This is one of the simplest mLAMP approach reported since identification of specific LAMP product was not needed. In this study, LAMP primer sets were specifically designed for each serotype of the dengue virus: DENV-1, DENV-2, DENV-3 and DENV-4 targeting on the 3′-noncoding region (NCR). Interestingly, all the primer sets were added together into a single tube of LAMP reaction to detect the presence of the dengue virus within 60 min of a LAMP reaction. The samples used in this reaction was the serum of dengue infected patients and healthy donors. Colorimetric detection was performed by using hydroxynaphthol blue (HNB), in which it allows the naked eye observation of final LAMP products. The sensitivity of this RT-mLAMP system was 10 RNA copies for all serotypes, and there was no cross-reactivity with three closely related arboviruses observed (Lau *et al.* 2015).

Due to formation of multiple amplicons in the mLAMP products, this method needs to be coupled with several types of multiplex product detection method to specifically differentiate them. One of the example is combining mLAMP with dot enzyme-linked immunosorbent assay (dot-ELISA) for differential identification of *Taenia solium*, *Taenia saginata* and *Taenia asiatica*, where cytochrome c oxidase subunit 1 gene was chosen as the target (Nkouawa *et al.* 2016). In this study, a single tube of mLAMP reaction was performed with FIP primers labelled with fluorescein isothiocyanate (FITC), digoxigenin (DIG) and tetramethylrhodamine (TAMRA), and BIP primers labelled with biotin. The mLAMP products from each plasmid containing the cox1 genes for *T. solium*, *T. saginata* and *T. asiatica* were captured by

antibodies that were specific for FITC, DIG and TAMRA, respectively. Following this, Strep-Tactin alkaline phosphatase conjugate and chromogenic substrate was used to detect the mLAMP products. A positive result was indicated by purple dots developed on the membrane while uncoloured dots represented a negative result permitting the differential detection of these species.

A study by Lee *et al.* (2010) reported the development of a one-tube reaction by using LAMP-PCR, hybridization, digestion with restriction endonuclease and colorimetric method of ELISA for the detection of *M. tuberculosis* that resistance to isoniazid, ethambutol and streptomycin. This was made possible by targeting hot spot point mutations in targeted drug-resistant genes: for isoniazid at katG 315 and katG 463, ethambutol at embB 306 and embB 497, and streptomycin at rpsL 43. In a reaction tube, LAMP primer sets targeting each of the mentioned point mutations and specific internal primers for PCR of individual resistance genes were added together with mycobacterial DNA template. The mixture was incubated at 65°C for 30 min to perform LAMP reaction followed by PCR assay by using LAMP products as the template. For differentiation of wild-type codons of resistance genes from mutant codon, specific restriction endonucleases were added to LAMP-PCR products where wild-type products were digested by specific restriction endonucleases. The digested products were then subjected to ELISA procedure where colour development and OD readings were recorded to indicate positive amplification for each target (Lee *et al.* 2010).

A rapid LAMP and real-time monitoring procedure was combined for the simultaneous detection of food-borne disease pathogens, such as *Salmonella* spp. and *Vibrio parahaemolyticus*, as reported by Liu *et al.* (2017). In a reaction tube, primer sets targeting these two species, respectively, were added and subjected to LAMP reaction for 60 min. However, LAMP selection caused by amplification bias was observed where *Salmonella* target sequence amplification rate is higher over that of *V. parahaemolyticus*. In order to overcome this issue, concentration of primer set detecting *Salmonella* was halved which resulted in equal amplification efficiency of both target sequence. After the isothermal amplification reaction, the products were subjected to a melting curve analysis and difference in melting temperatures (T_m values) during the LAMP reaction was observed to distinguish these two pathogens (Liu *et al.* 2017).

A novel mLAMP was developed by Stratakos *et al.* (2017) for detection and differentiation of nonpathogenic *Escherichia coli* and verocytotoxigenic *E. coli* (VTEC) in beef and bovine faeces. The mLAMP assay was designed for the detection of generic *E. coli* (nonpathogenic) targeting the *phoA* gene and VTEC strains targeting the *stx1* or *stx2* genes at 65°C for 30 min. In terms of the assay specificity, no cross-reactions were reported when 58 bacterial strains and specimens was used. Limit of detection for this assay was reported to be 10^3–10^2 CFU per g, which may be adequate to be used for monitoring hygienic status of beef and to control food-borne diseases caused by VTEC (Stratakos *et al.* 2017).

The utility of mLAMP for detection of multiple genes for specific identification of parasites in mosquitoes was reported by Aonuma *et al.* (2010). In this reaction, a mixture of LAMP primers for the detection of SPECT2 gene of *Plasmodium berghei* and cytochrome oxidase subunit I gene of *Dirofilaria immitis* was used. The FIP for each primer set was specifically labelled with FITC for *P. berghei* and cyanine5.5 (Cy5.5) for *D. immitis*. Real-time turbidity metre was used to monitor the amplification reaction and image analyser was used to examine the fluorescent dye-conjugated fragments. Green fluorescence signal produced by FITC indicated positive amplification of *P. berghei*, while for *D. immitis*, a magenta colour of Cy.55 was observed. This permitted the simultaneous detection of these two-parasitic species (Aonuma *et al.* 2010).

Electric LAMP

A study reported by Salinas and Little (2012) explains on the development of electric LAMP (eLAMP), an electronic simulation where it provides a fast and inexpensive putative tests of LAMP primers on target sequences compatibility. This method helps to improve the efficiency and aids to determine the opportunity of using existing primers to detect recently discovered sequence variants (Salinas and Little 2012). In this study, sets of existing LAMP primers were tested using this eLAMP on 40 whole-genome sequences of *Staphylococcus.* The results correctly predicted that the tested primer sets would amplify from *Staphylococcus aureus* genomes and not from other *Staphylococcus* species.

In-disc LAMP

Santiago-Felipe *et al.* (2016) developed an assay for detection of pathogenic *Salmonella* spp. and identification of bovine meat in meat samples by using the principle of in-disc LAMP (iD-LAMP) and quantitative optical readout by a disc drive. iD-LAMP is an integrated device composed of micro-reactors embedded onto compact discs for real-time targeted DNA determination (Santiago-Felipe *et al.* 2016). Similar reagents used in conventional LAMP were used in this iD-LAMP except that it was performed in a micro-reactor placed in a 65°C oven. During the incubation, the disc was cyclically scanned

and a sample was considered positive when the optical response was greater than the cut-off value. From this study, it shows that the result of iD-LAMP was comparable to those obtained by conventional LAMP with an extra advantage of using a smaller amount of template.

LAMP end-point detection method

Frequently used detection methods include turbidity, agarose gel electrophoresis, calorimetric detection using naked eyes and detection using UV light. Real-time monitoring based on turbidity using real-time turbidity metre (e.g. Loopamp Realtime Turbidimeter LA-200, LA-320, LA-500, Eiken Chemical Co., Ltd., Tokyo, Japan) is commonly used for LAMP product detection. LAMP assay was monitored for the white precipitation caused by the presence of magnesium pyrophosphate, $Mg_2P_2O_7$ (a by-product of LAMP reaction) at optical density 650 nm every 6 s. Several studies have been reported using turbidity to detect the presence of desired micro-organisms, for instances, *Mycoplasma pneumoniae* (Sakai *et al.* 2017), *Salmonella* spp. (Mashooq *et al.* 2016), human papillomavirus (Kumvongpin *et al.* 2016) and *Clostridium difficile* (Lin *et al.* 2015). In case of unavailability of turbidimeter, a brief centrifugation of the LAMP products could be used as an alternative to observe the white precipitation, which is visible to naked eyes (Zhou *et al.* 2014).

Agarose gel electrophoresis could be used to visualize LAMP products as well. The end products of LAMP gene amplification will be stem-loop DNA structures with several inverted repeats of the target and cauliflower-like structures with multiple loops which cause the gel electrophoresis image of these products to be in ladder-like patterns. This method has been used for detection of *E. histolytica* (Mwendwa *et al.* 2017), *S. aureus* (Chen *et al.* 2017a), *V. parahaemolyticus* (Siddique *et al.* 2017), *Clostridium perfringens* (Radhika *et al.* 2016), parasitic tapeworm *Echinococcus* spp. (Ahmed *et al.* 2016), and plant disease caused by Southern tomato virus (Elvira-Gonzalez *et al.* 2017). However, visualization of LAMP DNA product by gel electrophoresis required the opening of the reaction tubes, therefore increases the risk of carry-over contamination, and application of gel electrophoresis is restricted due to the use of ethidium bromide stain, a highly hazardous chemical which is carcinogenic to human (Zhou *et al.* 2014).

An alternative to gel electrophoresis, intercalating dyes were used for direct detection of the DNA products, for example, calcein, SYBR green I and HNB dye. Calcein dye, a metal ion binding fluorophore, which was added before isothermal incubation will form an insoluble salt complex, manganese-pyrophosphate (manganese ion, Mn^{2+} from calcein whereas pyrophosphate is the by-product of LAMP reaction), thus making the reaction fluorescent. Subsequently, the fluorescence is further intensified when free calcein binds to magnesium ions (Mg^{2+} from LAMP reaction mixture) and could be easily be observed under UV light (365 nm; Sahoo *et al.* 2016). The reaction between Mg^{2+} and calcein also causes the colour changes from orange to green, which could be observed via naked eyes under natural light. Positive LAMP reaction was indicated with green colour, whereas negative LAMP reaction indicated with orange colour. To name a few, calcein has been used in detection of *Trypanosoma cruzi* (Besuschio *et al.* 2017), avian influenza H5 virus (Liu *et al.* 2013), hepatitis C virus (Zhao *et al.* 2017), human papillomavirus (Lin *et al.* 2017), human coronavirus (Geng *et al.* 2016) and *Flavobacterium columnare* in tilapia fish (Suebsing *et al.* 2015). Calcein-based end-point detection is most commonly used due to its high sensitivity and notable colour change for naked eye observation (Fischbach *et al.* 2015).

SYBR green I is also commonly used in LAMP assay. Addition of SYBR green I to reaction tubes containing LAMP products lead to visible colour change from reddish orange to yellowish green, and fluorescent under UV light. SYBR green I has been used for detection of coxsackievirus (Monazah *et al.* 2017), *Plasmodium vivax* and *Plasmodium falciparum* (Singh *et al.* 2017), *Mycoplasma synoviae* (Kursa *et al.* 2015), fish pathogen *Nocardia salmonicida* (Xia *et al.* 2015), maize chlorotic mottle virus (Chen *et al.* 2017b) and tomato chlorosis virus (Zhao *et al.* 2015). Despite reported with good sensitivity, SYBR green I dye could inhibit LAMP reaction if the dye is added before isothermal incubation. Post-LAMP added dye required the opening of the LAMP tube which will leads to false-positive results due to aerosol contamination (Karthik *et al.* 2014). HNB dye was commonly used as well for visualization of LAMP products. HNB dye changes from violet to sky blue colour and has been used for identification of Gram-negative bacilli (Srisrattakarn *et al.* 2017), *Chlamydia trachomatis* (Choopara *et al.* 2016), and *M. tuberculosis* (Balne *et al.* 2015), orf virus in goat and cattle (Inoshima *et al.* 2017) and feline coronavirus (Stranieri *et al.* 2017). Addition of HNB dye in LAMP reaction tube prior amplification could help to reduce aerosol contamination to the surrounding; however, HNB does not exhibit fluorescent ability, thus, only limited to naked colour changes observation (summarized end-point detection in Fig. 2).

Other dyes such as malachite green dye (Sriworarat *et al.* 2015; Lucchi *et al.* 2016; Nzelu *et al.* 2016), Picogreen dye (Batra *et al.* 2015), Gelred dye (Wozniakowski *et al.* 2013; Wassermann *et al.* 2014), SYTO fluorescent dye (Liu *et al.* 2013; Watts *et al.* 2014; Mwendwa *et al.* 2017), Evagreen dye (Lee *et al.* 2016; Oscorbin *et al.*

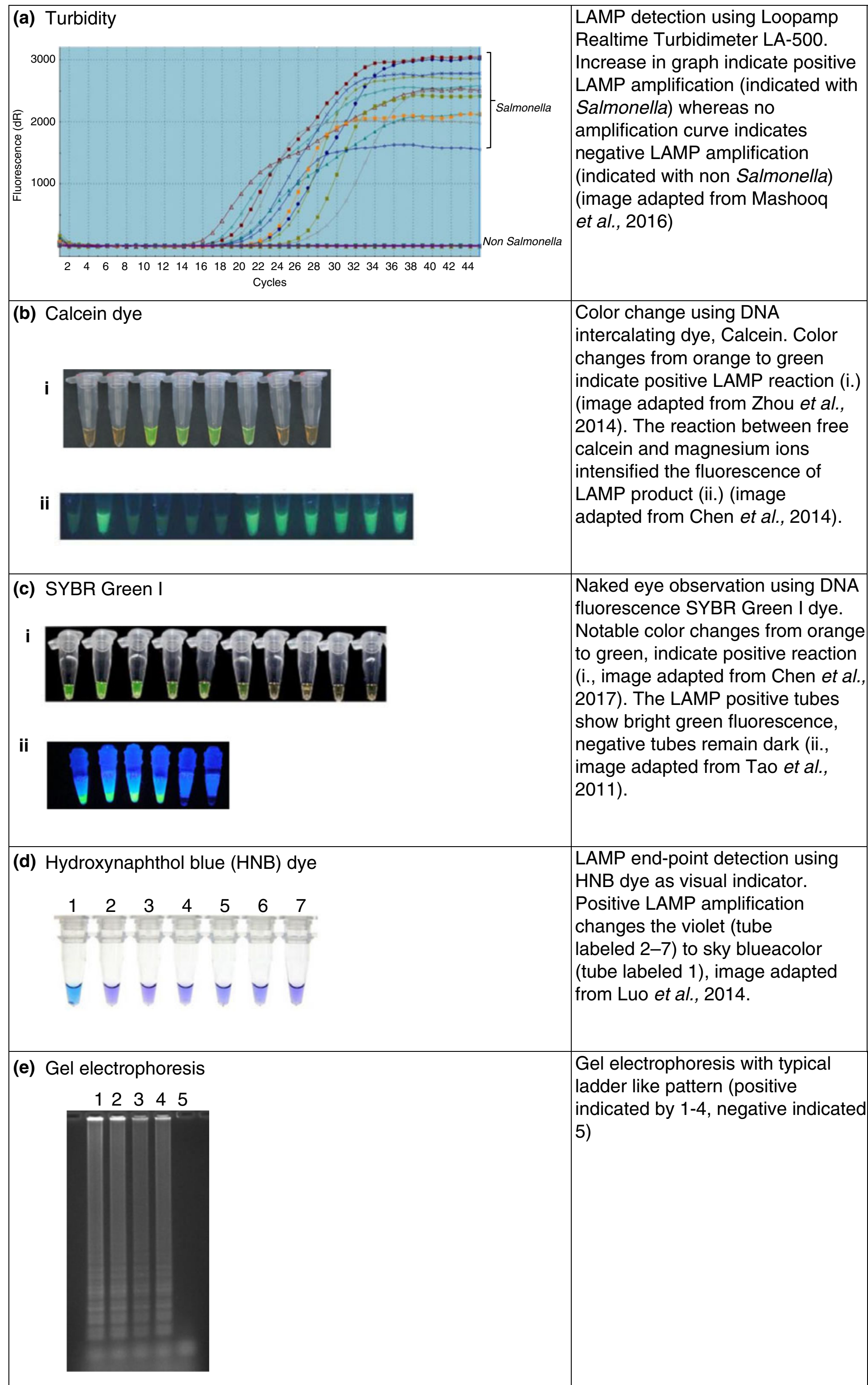

Figure 2 A summary of the commonly used LAMP detection method. (a) LAMP endpoint detection with turbidity, (b) LAMP calorimetric detection using calcein dye, (c) LAMP calorimetric detection using SYBR Green I dye, (d) LAMP calorimteric detection using HNB dye, and (e) analysis of LAMP product using gel electrophoresis. [Colour figure can be viewed at wileyonlinelibrary.com]

2016), and Goldview II dye (Shi *et al.* 2011; Wu *et al.* 2014) were used as well for detection of LAMP DNA products. An alternative natural plant alkaloid dye known as berberine has been explored and used for detection of LAMP products. A study has been reported that berberine dye exhibit similar specificity and sensitivity compared with existing DNA intercalating dye and can be used as a fluorescent probe. Berberine dye is recommended for large field applications as it is cheaply available than other dyes (Fischbach *et al.* 2015).

Recently, more studies have been focused on developing lateral flow assay (LFA) for end-point detection of LAMP product. LFA is currently a favoured technique due to low-cost, user-friendly, simple, rapid, light and portable. The sample of interest, for example, saliva, blood, urine and other body fluids, is applied at one end of the strip (sample pad), which is conjugated with LAMP buffer mix and biotin, making the loaded sample suitable for interaction with the detection system. Most generally, lateral flow test was established using fluorescein in isothiocyanate (FITC) labelled DNA probes (which will recognize the specific region in the LAMP amplicon) hybridized with biotinylated LAMP amplicons (Thongkao *et al.* 2015; Fowler *et al.* 2016; Huang *et al.* 2017; Park *et al.* 2017). This complex would be captured by streptavidin on the biotin, forming another complex with anti-FITC antibody coated on the gold nanoparticles. This would give a visible result on the test line. However, the biotinylated nontargeted product would not hybridize with FITC DNA probe (only specific to LAMP amplicon), nor forming complexes with gold-labelled anti-FITC antibody to react with streptavidin on the test line, generating no signal thus a negative result on the test line. This complex would then move through the test line and was trapped at the control line (Chowdry *et al.* 2014; Yan *et al.* 2015; Nawattanapaiboon *et al.* 2016).

Successful LFA includes multiplex LFA-LAMP to simultaneously detect *sea* and *seb* gene of *S. aureus* (Yin *et al.* 2016) and identification of adulterants in meats (Li and Fan 2016). Lateral flow dipstick (LFD) was developed as well for simultaneous detection of pathogenic *Leptospira* spp. (Nurul Najian *et al.* 2016), measles virus (Xu *et al.* 2016), animal babesiosis, caused by *Babesia bovis* and *Babesia bigemina* (Yang *et al.* 2016), foot-and-mouth diseases (Waters *et al.* 2014), *Japanese encephalitis* (Deng *et al.* 2015), *P. vivax,* and *P. falciparum* (Yongkiettrakul *et al.* 2014; Kongkasuriyachai *et al.* 2017), Jembrana disease virus (Kusumawati *et al.* 2015), canine parvovirus (Sun *et al.* 2014), *Vibrio alginolyticus* (Plaon *et al.* 2015), *Macrobrachium rosenbergii* nodavirus (Lin *et al.* 2014) and shrimp yellow head virus (Khunthong *et al.* 2013). Lateral flow biosensor (LFB) was successfully developed for multiplex detection of *Enterococcus faecalis* and *S. aureus*. The development of the LFB with gold nanoparticles had greatly helped in the visual detection of human pathogens without any instrumentation (Wang *et al.* 2017).

Further advancement of LAMP end-point detection was made when Draz and Lu (2016) developed LAMP coupled with Surface Enhanced Raman spectroscopy (SERS) for rapid detection of *Salmonella enteritidis*. The study reported SERS detection labelling with Raman active gold nanoparticle was highly sensitive (100 times more than PCR), requires little sample preparation, and improves specificity and reliability by limiting false-positive results (Draz and Lu, 2016).

Advances in LAMP

Moving towards the application of LAMP as a point-of-care detection method, several advances have been reported to date including development of lyophilized form of LAMP reagents and commercially available LAMP kit for micro-organisms' specific detection.

Lyophilized LAMP is a term used for the dried form of LAMP reagents, where it can withstand ambient temperature and long storage before being used for any molecular detection purpose hence the making of on-site diagnosis is possible. One of the examples on the utilization of lyophilized LAMP was reported by Hayashida *et al.*, where they developed a single tube of dried LAMP reagent for diagnosis of human African trypanosomiasis on-site. By using this lyophilized LAMP reagents, the detection limit for detection of repetitive insertion mobile element (RIME), 18S rRNA and SRA-LAMP were 0·01, 0·1 and 1 parasite equivalent DNA per reaction, respectively. In terms of thermostability assessment, the lyophilized LAMP was incubated at 25 or 45°C for 72 h and used in comparison with fresh LAMP reagents for RIME-LAMP reaction, where the result was comparable (Hayashida *et al.* 2015).

The application of lyophilized LAMP reagents was reported by Chen *et al.* (2016) for the detection of *Leptospira* by using a LAMP primers combination targeting *lipL32* and *lipL41* genes. *Bst* DNA polymerase, dNTPs and primers were supplemented with sugars and subjected to lyophilization in a freeze-dryer. To evaluate the reagent stability and sensitivity, lyophilized LAMP reagents, which were stored at 4, 25 and 37°C, were used to test the detection limit alongside the freshly prepared LAMP reagents for *Leptospira* detection. The limit of detection observed for these two preparations were 12 copies of genomic DNA. In terms of stability, the results showed that lyophilized LAMP reagents retain the same reactivity as freshly prepared reagents and remain stable

for up to 3 months when stored at 4°C (Chen *et al.* 2016).

One of the latest development of the lyophilization method of RT-LAMP reaction was reported by Carter *et al.* (2017) with its application in the detection of Zaire Ebola Virus (ZEBOV). LAMP primers targeting *L* gene of ZEBOV were designed based on the alignment of isolates from 1976, 1995, 2007 and 2014 outbreaks as well as corresponding fragments were synthesized and cloned into plasmid which served as the template for in vitro transcription of the sense strand. The master mix was then subjected to cool in liquid nitrogen followed by freeze-drying. In this study, they highlighted key modifications of the reaction components in order to maintain the lyophilized reagents stability, which was the addition of a nonreducing sugar trehalose and dialysis of the used enzyme preparations (Carter *et al.* 2017).

Several LAMP kits are commercially available in the market. For instance, the Loopamp TB detection kit, *Trypanosoma brucei* kit, *L. monocytogenes* detection kit, *Legionella* screening kit E for environmental detection, Malaria kit, *SARS Coronavirus* detection kit, *Campylobacter* detection kit, *M. pneumoniae* detection kit, *E. coli* O157 detection kit, *Cryptosporidium* detection kit, and Bovine Embryo Sex Typing Kit are all currently available from Eiken Chemical Co., Ltd. In 2010, another LAMP assay to detect *C. difficile* was developed by Meridian Bioscience USA, termed as *illumigene*® *C. difficile*. It was welcomed by many researchers due to its high sensitivity for detecting *C. difficile* (Pancholi *et al.* 2012).

LAMP primer sets are also commercially available from Eiken Chemical Co., Ltd, such as the Loopamp primer set for the West Nile virus; *Avian* flu H5 and H7; *FluA* influenza; *Flavobacterium psychrophilum* and *Alicyclobacilus acidoterrestris*.

Advantages of LAMP

As suggested by WHO, the criteria for an ideal diagnostic test must comprise sensitivity, specificity, low-cost, simplicity, rapidity, adaptability to all kinds of climatic changes and the availability of instruments (Mabey *et al.* 2004). Since detection of diseases is becoming progressively more difficult due to the occurrence of new as well as existing disease or pathogens, an alternative diagnostic method is in need to be discovered. Among the amplification techniques, LAMP stands out an effective diagnostic test. It is a preferred diagnostic tool, even though its principle and reaction mechanism is slightly complex (Notomi *et al.* 2015). Ever since its development, the striking properties of LAMP have encouraged researchers to further explore its usage for analysis in various fields.

As been discussed in this paper, LAMP could be one of the diagnostic method alternative in relation to its rapidity, sensitivity (Ishiguro *et al.* 2015; Kumvongpin *et al.* 2016; Besuschio *et al.* 2017) and specificity. The most significant advantage of LAMP would be its rapidity where LAMP allows immediate diagnosis. Unlike any other nucleic acid amplification technique, for example, PCR, LAMP could be performed in just 30 min rather than at least 90 min for PCR. In addition, a LAMP amplification proceeds rapidly since the initial heat denaturation for the DNA template is not required.

Another advantage of LAMP would be its simplicity to be performed where all it needs is a simple water bath or heating block that provides an isothermal condition. This eliminates the need of advanced instruments as needed in other amplification technique. LAMP has the potential to improve molecular biology technology with the simplicity of the assay as it is much easier to use as compared with PCR. The simplicity of LAMP can also be described in the method of analysing the results. Direct evaluation of results were made possible whereby the dyes used in LAMP reactions enable naked eye observation of colorimetric changes be it under natural light or UV light with the use of fluorescence dye. Thus, unlike PCR, tedious gel electrophoresis procedure of the amplification products is not needed.

Apart from that, a new pH sensitive dye for LAMP was recently developed by taking into consideration the unique features of *Bst* DNA polymerase in buffered solutions. Since a significant pH change from alkaline to acidic was observed during LAMP amplification, pH indicator dyes can be used to monitor the LAMP reaction and a significant colour change was easily visualized (Tanner *et al.* 2015). This helps to further improve the flexibility of LAMP as a simple tool for use in endemic countries. LAMP assays also appeared unaffected by the presence of nontarget DNA in the acquired samples (Kaneko *et al.* 2007; Lin *et al.* 2012) which resembles its high specificity characteristic. The LAMP assay is stable against some PCR inhibitors such as blood and detection can be performed without the template extraction step and with a nonprocessed sample (Nagamine *et al.* 2001; Francois *et al.* 2011; Lin *et al.* 2012; Mori *et al.* 2013; Wang *et al.* 2013).

As LAMP advances, it can be made into a portable device to ease transportation to the site of diagnosis, for example, the diagnosis of the Ebola virus and food-borne pathogens (Uddin *et al.* 2015; Kurosaki *et al.* 2016; Safavieh *et al.* 2016). On top of that, LAMP can amplify RNA with high efficiency by using reverse transcriptase (Mori *et al.* 2001; Nagamine *et al.* 2002; Dukes *et al.* 2006). At present, improved DNA polymerases, such as *Bst* 3.0 polymerase and OmniAmp polymerase, are

available for the application of LAMP and RT-LAMP. These DNA polymerases demonstrated a robust performance with increase reverse transcriptase activity as compared to conventional *Bst* DNA polymerase (Chander *et al.* 2014; Tian *et al.* 2016).

Limitations of LAMP

Despite the robustness of this novel technique, there are a few limitations that could impede its smooth application. This technique is inapplicable for cloning or other molecular biology purposes since the final product is a large DNA chain, making it to be less versatile when compared to PCR (Sahoo *et al.* 2016). Furthermore, the multiplexing approaches of LAMP is still considered less successful than PCR as they increase the difficulty of the experimental design and procedures (Dhama *et al.* 2014).

It is also challenging to choose a correct and proper target for amplification, for either a highly conserved region or target site which is specific to a particular strain of micro-organism. Furthermore, in order to increase the sensitivity and specificity, LAMP uses 4–6 primers targeting 6 or 8 regions within a rather small segment of target sequence. Therefore, the primer design is exposed to many restrictions and it is problematic to design a LAMP primer. Even though a free online software is available to help with primer design, however, under certain circumstances, some of the preferred target sites might not be selected and, thus, would still rely on the need to manually design the primers. The usage of multiple primers will increase the chances of primer–primer hybridizations leading to template-free amplification, giving false-positive results (Watts *et al.* 2014), but the chances of false positivity require further investigation. In the case of the aforementioned phenomenon occurs, it is recommended to redesign the primers.

One major drawback of LAMP is its high risk of carryover contamination which often leads to false-positive results in negative controls (Hsieh *et al.* 2014; Karthik *et al.* 2014). This is due to the extremely high efficiency of the LAMP reaction. The LAMP product is so stable that it is not degraded easily and thus unintended carryover contamination can occurs (Bi *et al.* 2012; Dhama *et al.* 2014; Fischbach *et al.* 2015). In case aforesaid contamination occurs, it is suggested to perform LAMP in a laminar flow hood and use separate pipettes and filtered tips. For procedures that involved opening of the reaction tubes, for example, gel electrophoresis, it is wise to carry out LAMP in an isolated room as LAMP is highly sensitive to even minute amounts of product contamination.

The turbidimetric and colorimetric determination of the LAMP reaction is subjective when determined by visual observation alone, as it relies on an individual's perception of colour (Bista *et al.* 2007). Furthermore, the product of LAMP results in a ladder pattern or smearing on the gel rather than a single band as in PCR, thus identification of a target by size of the band on a gel is not possible with LAMP (Wastling *et al.* 2010).

Future potential of LAMP

The significant characteristics of LAMP are easy handling – it only needs a simple water bath at a constant temperature and its rapidity. Developing a mobile biosensor with LAMP technology application may result in a significant advancement. To date, LAMP was widely used as a diagnostic tool for the detection of human, livestock and plant diseases. However, there are few publications on the use of this technique for the direct detection of environmental pathogens. The development of a sensor that can detect the presence of pathogenic pathogens directly from an environmental sample may bring vast benefits, especially in diseases that are caused by environmental pathogens, such as Leptospirosis. Apart from that, LAMP could also be applied in the early detection of genetic diseases in a foetus, for instance, Down syndrome and thalassemia, as it has high specificity. The development of microfluidic devices with LAMP also comes with many advantages, such as easy to operate, palm-sized, high output applicability, decrease in the consumption of expensive reagents and do not require electricity. LAMP microfluidic devices are commonly developed for food testing, cancer screening and virus detection. In summary, all of the features of LAMP prove that its straightforwardness is beneficial for the on-site field or diagnostics in an everyday basis in a POC setting.

Conclusion

LAMP is a ground-breaking gene amplification technique. It works as a fast and simple diagnostic tool for the quick detection and identification of microbiological infections. Besides being simple to operate and easily adapted to any field circumstances and environments, LAMP has all the features required for real-time assays, especially in high sensitivity. LAMP is also a beneficial and valuable tool for use in developing countries due to its ease of performance without needing any advanced equipment or experts to operate it. In summary, LAMP could be the ideal diagnostic method, as it fulfils all criteria proposed by the WHO on ideal diagnostics.

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Conflict of Interest

No conflict of interest declared. All authors read and approved the final version of the manuscript.

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