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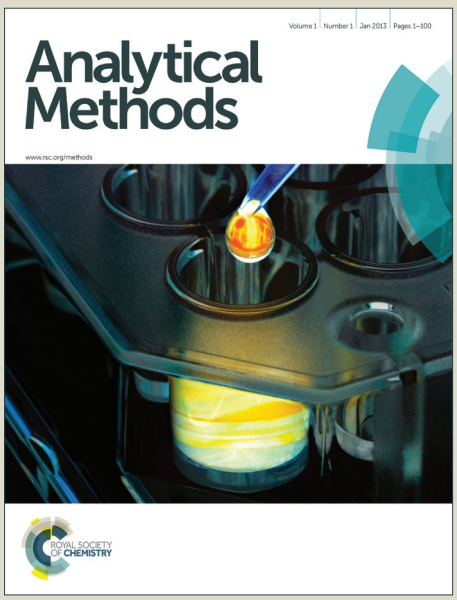

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Advances in Loop-mediated Isothermal Amplification: integrated with several point-of-care diagnostic methods

Pin Gong[a,a*], Taotao Zhang[a,a], Lan Wang[a], Fuxin Chen[b], Sai Jin[a] and Xiaohuan Bai[a]

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Recent outbreaks linked to pathogenic bacteria heighten the need to develop rapid, sensitive, portable and low-cost pathogen diagnostic systems. Loop-mediated isothermal amplification (LAMP) is a powerful and novel gene amplification method emerging as a simple rapid diagnostic tool for early detection and identification of microbial disease. Herein, we highlight the recent advances concerning LAMP which is integrated with several innovative detection technologies, thus, this article also reviews the extensive application of these basic techniques in food-borne bacterial detection.

Introduction

To our knowledge, bacterial infection is one of the most common causes of death in developing countries, accounting for about 40% of deaths [1]. Even in the United States each year about 1 in 6 gets sick, over 300,000 are hospitalized, and more than 5000 people die of food-borne diseases caused by pathogenic bacteria [2].Thus, it's necessary to put forward some effective food-borne bacteria detection methods in order to strengthen the surveillance and control of acute infectious microbial disease.

Generally, many agencies in the world mainly use microbial identification techniques for pathogens detection, which are labor-intensive and time-consuming, demanding several days for a final result. Usually, pathogens will not be detected until the products are available to consumers, causing outbreaks and infections in general population. Immunoassays such as enzyme-linked immunosorbent assay have been developed for the detection of bacteria. However, their uses have been limited by the low specificity because of cross-reaction. Molecular diagnostic methods can refrain from the disadvantages of present tools and improve the efficiency of pathogen diagnosis. Many molecular biology-based methods have been developed for the detection of food-borne bacteria such as PCR, real-time PCR and situ-PCR [3]. These methods are rapid and sensitive; however, the application of these assays is restricted due to their need for expensive or sophisticated equipment and highly trained technicians. Also, the traditional extraction of DNA takes more than 1 hour, and the PCR, real-time PCR all may take long circulation times, disordered electrophoresis, and ultraviolet observance. Compared with these methods listed above, isothermal amplification methods such as loop-mediated isothermal amplification (LAMP) can be performed in a simple incubator by unskilled personnel and applied to point of care(POC) testing. Due to its advantages of rapid amplification, simple operation and high sensitivity and specificity, LAMP has been widely used as a promising alternative to detect bacteria and virus in food, diagnose and environment. Unfortunately, several

[a] a.College of Life Science and Technology, Shaanxi University of Science and Technology, Xi'an, China. *Fax:+86-29-86132711; Tel:+86-29-86132711; Email:lavenderpin@gmail.com*
[b] School of Chemistry and Chemical Engineering.Xi'an University of Science and Technology. Xi'an.China.

factors including false-negative results caused by environmental contaminant and aerosol, the application of some carcinogens reagents in electrophoresis analysis of amplicons, prevent its true practical application.

More recently, the combination of LAMP with new detection techniques has been developed. They are mainly portable, rapid, specific and sensitive, and effectively avoid the deficiency of single LAMP assay. Also, DNA extraction method may be improved by combining with magnetic-bead technology. Thus genetic detection of bacteria or virus at the POC has become increasingly important either in molecular diagnostics or food safety detection [4], which could support real-time, semi-quantitative or quantitative analysis and offer information through computer or other devices which conventional end-point detection methods fail to provide [5].

Brief introduction of LAMP assay

LAMP was firstly described and initially evaluated for the detection of hepatitis B virus DNA in 2000, which is a typical innovative gene amplification technique for detection and identification of microbial diseases [6].This method is characterized by application of six specifically designed primers to recognize a total of eight distinct sequences on target DNA.The principle of LAMP has been illustrated in Figure 1.

The amplification of DNA proceeds with high specificity under isothermal condition within 60 min, and the reaction can be monitored by detecting the increase in turbidity of reaction mixture [7], agarose gel electrophoresis [8] as well as color change following addition of SYBR-Green I [9] and calcein reagent [10]. So when it comes to the advantages of LAMP, rapid, stable and sensitive detection of pathogenic bacteria is critical for disease control. Compared to PCR and real-time PCR, LAMP has the advantages of no need to heat and denaturation of template DNA [11], and then gains popularity among researchers due to its simple operation and rapid reaction for easy adaptability to field conditions. However, some disadvantages of the assay also need to be considered, such as false-negative results caused by environmental contaminant, convenient, feasible and quantitative synchronization testing for practical purposes in diagnostic laboratories or in field-testing. Therefore, it's necessary to emphasize the extensive application of LAMP assay.

Further research on LAMP and its advantage has facilitated the application of LAMP technology in practical use. For example, Edwardsiella tarda from infected Japanese flounder has been detected by LAMP-based assay, targeting the haemolysin gene [12]. More recently, two steps reverse transcription LAMP (RT-LAMP)assay has been used to detect the foot-and-mouth disease virus [13] and HIV-1 viral RNA [14]. Significantly, in Japan, several LAMP reagents have been approved, including SARS corona virus, Mycobacterium tuberculosis, influenza type A virus and so on. Moreover, the tuberculosis-LAMP reagent has been provided as dry powder, allowing easy storage at room temperature [15].

LAMP integrated with POC methods

Brief introduction of POC

POC assay is to establish an ease-of -use, portable and quantitative testing for on-site screening of multiple pathogens relevant to food safety, effectively to resist contamination and complete quantitative detection. POC diagnostics critically employ

miniaturization and combination of sample processing, nucleic acid amplification, and detection systems[16]. In order to develop sample-in-answer-out genetic analytic methods, both PCR and isothermal assays (e.g. LAMP) have been translated on micro/microfluidic chips [17-18]. However, POC-PCR is performed on more complex and expensive devices, causing more limitation under resource limited institutions. While POC-LAMP is considerably cheaper than systems with PCR-based assays, and they are relatively more adopted by commercial application. Fang et al.[19] established an octobus-like microfluid chip combining LAMP assay to simultaneously detect several viruses in one setting. The assay result may be directly determined via the insoluble byproduct magnesium pyrophosphate. Generally, the genetic marker-based POC diagnostics include the following advantages: 1) miniaturized nucleic acid amplification; 2) shorter analysis times; 3) reducing reagent consumption; 4) minimized risks of sample contamination; 5) enhanced assay performance.

LAMP-lateral flow dipstick (LAMP-LFD)

Traditionally, analysis of LAMP products (amplicons) is usually carried out by agarose gel electrophoresis, followed by ethidium bromide(EB) staining and observation in the presence of fluorescent intercalating dye SYBR Green I. Non-specific amplification products may cause false positive results and the use of EB may lead to some other risks. In order to eliminate electrophoresis-involved EB and lower the false positive results, generic lateral flow dipstick (LFD) strips (Milenia® GenLine HybriDetect) were employed. Briefly, after the regular LAMP reaction under optimistic conditions except heat inactivation, the FITC probe was added into the LAMP reaction tube and incubated at 63℃ for 5 min. Finally, the above mixture was mixed with the assay buffer, and the LFD strip was dipped into it to detect the hybrid of amplicon-probe. These strips detect biotin labeled LAMP product that has been hybridized with an FITC-labeled DNA probe complexed with gold-labeled anti-FITC antibody. The resulting triple complex is trapped at the test line, while the negative result is trapped at the control line(Figure 2.). The LFD does not require special instrument since the user can simply dip the LFD strip into an appropriately buffered LAMP. Using the combined LAMP–LFD[20], total assay interval was approximately 65 min, excluding DNA extraction time, and detection sensitivity was near that of other common-used methods ,such as nested-PCR and real-time PCR. In conclusion, the assay has all the required advantages (high sensitivity, specificity, relatively short analysis time, and no need of a thermocycler). However, this method could not provide quantitative analysis of the amplified products.

Fluorescent resonance energy transfer (FRET) probe technology

One of the conventional analysis of DNA amplicons is to monitor the precipitation formed by its reaction byproduct such as magnesium pyrophosphate [21], but several researchers have proved that the increased turbidity of the amplified products due to the precipitation of magnesium pyrophosphate during the amplification process [22]. A DNA yield of ≥4 μg may push the pyrophosphate ion concentration above 0.5 ppm，and people can observe turbidity in form of a white precipitate[23]. So it's difficult to detect the amplicons, if the amount of the magnesium pyrophosphate can't be controlled properly. Aiming to detect the amplicons effectively and avoid the time-consuming process such as agarose gel electrophoresis, EB staining, FRET probe technology were employed. During the process, FRET probes designed to target loop region of the LAMP amplicons were added to conventional LAMP reagent. The mixed LAMP reaction was carried out in LightCycler capillary tube at 90℃ for 60-120min and terminated at 90℃

for 2min. Finally, the amplicons will be illuminated at 495nm (excitation wavelength) and 640nm (emission wavelength), and the result may be showed in the software （Figure 3.）.

Chou et al.[24] combined FRET probe with target loop region on LAMP amplicons to detect the white spot syndrome virus. After optimization of the experiment, they reduced reaction cycle to less than 70 min without any cross activity. In 2006, Mori et al. [25] amplified the target gene in the presence of fluorochrome-labeled oligo-DNA probe. Then, the polyethylenimine was added into the mixture, resulting in precipitation of LAMP amplicon-polyethylenimine complex and hybridization of fluorescently labeled Oligo DNA probes with LAMP product. The color of emitted fluorescence can be detected easily by naked eye on a conventional UV illuminator.

In conclusion, the real-time FRET assay does not require post-amplification, and products can be detected by naked eyes and fluorospectro photometer. Generally, FRET-LAMP assay has been proved to be specific, sensitive, and accurate for detection of food-borne bacteria with minimal risks of cross contamination. However, FRET probe is difficult to be designed and the process is longer than normal LAMP assay. The assay can be monitored by naked eyes or give a semi-quantitative result through fluorospectro photometer, and also cannot provide a quantitative analysis.

Magnetic-beads based micro-fluidic LAMP

Extraction of target DNA is the first step of molecular detection. Traditional extraction method is mainly based on the following four steps: cell lysis, extraction, isolation and purification, which are time-consuming. Also, target DNA is easy to be contaminated by some molecules such as proteins, RNA and so on. Magnetic-beads based micro-fluidic LAMP employs specific probe conjugated to magnetic beads, and target DNA can be specifically recognized and hybridized onto the surface of magnetic beads which are then purified and concentrated from samples, followed by nucleic acid amplification of target genes using LAMP. More importantly, the assay does not employ traditional agarose gel electrophoresis after the process of nucleic acid amplification, whereas, it provides a convenient way to detect amplicons using spectrophotometer and gives a semi-quantitative result（Figure 4.）.

Wang et al.[26] reported the assay for rapid detection of methicillin-resistant Staphylococcus aureus. Finally, the OD value was measured and the limit of detection was much lower than that of conventional PCR and real-time PCR. The use of various bulky apparatus and dye-labeled primers can be avoided by using absorption spectrophotometry. Significantly, this magnetic-bead based diagnostic assay may be more suitable in semi-quantitative analysis compared to those assays mentioned above. Also, the whole diagnostic process (from bio-sample pretreatment to optical detection) can be automatically completed within 60 min, traditionally, the extraction of target DNA may take over 60min.

In 2010, Wang et al. [27] also established a novel microfluid automatic assay for rapid detect viruses from tissue samples, combining targeted RNA extraction with a one-step RT-LAMP process. The whole process, from sample pre-treatment to target gene amplification, can be completed within 60 min in an automatic system without cross-reactions with other tested virus, bacteria and eukaryotic cells. So this assay may also become a powerful tool for rapid purification and detection of gram-positive bacterium, in which it is difficult to extract genomic DNA. Also, it's a potential POC platform for detecting other types of infections.

LAMP-electrochemical assay combined with a USB portable potentiostat

Some disadvantages of PCR, real-time RT-PCR and LAMP, for instance, the complicated detection process of amplified DNA and/or the false positive caused by the cross-reaction, restrict the practical application of these methods. Traditionally, the detection of amplicons was carried in miniature tube and LAMP reagent was easy to be contaminated. The electrochemical assay allows electrode inserting into 200μl micro-tube, and the whole amplification process can be monitored through the software, which can effectively refrain from the cross-reaction. In the process, electro active reagent methylene blue (MB) is required, which can be combined with the amplified DNA. Firstly, reverse transcription and DNA amplification can be accomplished at a constant temperature (63℃) in over 60min in one-step by the use of reverse transcriptase together with Bst DNA polymerase and several series of primers. Then, amplified DNA is combined with MB and the electrode surface may be covered with the complex leading to the reduction of peak current. The peak current monitored by square wave voltammetry using a USB portable potentiostat will decrease following the increased amount of amplified DNA.

Nagatani et al.[28] employed successfully semi-real time electrochemical monitoring for virus detection by reverse transcription loop-mediated isothermal amplification using a USB powered portable potentiostat. Wei et al.[29] reported that a satisfactory result was obtained using electrochemical DNA biosensor to detect LAMP product of *Yersinia enterocolitica* gene sequence in pork meat. This assay has also been used to test pathogenic DNA at POC through micro-fluidic electrochemical quantitative LAMP such as *Escherichia coli* [30] and marine bacterium *Vibrio parahaemolyticus* [31].

In summary, LAMP-electrochemical assay shows some advantages. First, the designed electrode can detect amplicons effectively and sensitively. Second, the assay can provide an effective full quantitative monitoring. So LAMP-electrochemical assay may provide all required advantages such as specificity, high-sensitivity, short-interval and fully quantitative analysis.

Conclusion

The integration of LAMP technology and innovative detection methods described in this review can effectively overcome several deficiency emerging in traditional assays which is limited in practical application of simple POC detection. Also, there are some relations among the assays described in the text. Compared to LAMP-LFD and LAMP-FRET which employ much simply instruments, but cannot provide a quantitative result, Magnetic-beads-LAMP and electrochemical-LAMP which need sophisticated apparatus can develop a POC quantitative detection for the purpose of bacteria detection in a wide range of fields.Compared to LAMP-LFD and LAMP-FRET which employ much simply instruments, but cannot provide a quantitative result, Magnetic-beads-LAMP and electrochemical-LAMP which need sophisticated apparatus can develop a POC quantitative detection for the purpose of bacteria detection in a wide range of fields.

Table 1 shows that Magnetic-beads based micro fluidic LAMP is convenient to get target purified DNA, especially for some gram-positive food-borne and some samples with rich nutritive matters, and the assay takes only 60min from the bio-sample pre-treatment, while other methods may take about 60 to 75 min to amplify and detect, excluding DNA extraction. In view of specificity and sensitivity, assays mentioned in the text are all up to the required level, and Magnetic-beads-LAMP and Electrochemical-LAMP have some advantages over the other assays. Significantly, FRET probe may limit the minimal risks of cross contamination, need no specific device and provide a semi-quantitative analysis. LAMP-electrochemical assay effectively combines the real-time USB portable potentiostat, which can cover the shortage of the above assays and provide a relative quantitative result, but the assay requires expensive electrode.

So it is better to integrate the merits of LAMP and some POC methods to develop an inexpensive portable device, termed as microfluidic LAMP for the rapid analysis of genes and point-of-care diagnostic. Successfully screening target DNA may be the first step to establish such a system, so we may get target DNA through magnetic-beads within several minutes.
Then for the purpose of automating POC detection, it will be promising to add FRET probes to traditional system to get a semi-quantitative result or connect traditional reaction mixture with USB portable potentiostat to get a quantitative result. That is, the combination will provide a real "sample-to-answer" assay for the effective detection of genes.

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Acknowledgement

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Table 1.Comparison of different LAMP-integrated methods from several aspects

	PCR	LAMP	LFD-LAMP	FRET-LAMP	Magnetic-beads-LAMP	Electro-chemical-LAMP
Analysis Time	~3h (excluding DNA extraction)	~60min (excluding DNA extraction)	~65min (excluding DNA extraction)	~75min (excluding DNA extraction)	~60min (containing DNA extraction)	~60min (excluding DNA extraction)
Post-Treatment[1]	+	+	+ +	-	+	-
Specificity[2]	+	+ +	+	+	+ +	+ +
Sensitivity	100± ng/ul	1± ng/ul	0.5±pg/ul	25±fg/ul	10± fg/ul	4± fg/ul
Facility requirement[3]	+	-	-	-	-	+
Quantitative analysis[4]	-	-	-	+ -	+	+ +

1. +, slightly need post-treatment; + +, greatly need post-treatment; -, no need post-treatment
2. +, high specificity; + +, strong specificity
3. +, high requirement of the facility; -,low requirement of the facility
4. +, quantitative result; -,non- quantitative result; +-,semi-quantitative result

Figure 1. Illustration of basic principle of LAMP

Figure1. Illustration of basic principle of LAMP

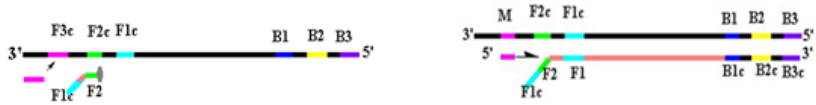
Step 1. starting material synthesis and cycling amplification stages;

During this process，a set of primers recognizing the six distinct sequences at the target DNA and Bst DNA polymarase are employed to hybridize the starting material, then the synthesis single-stranded DNA could be displaced by a new strand DNA primered by an outer primer, acting as template for the second inner primers and inner primers.

Step 2. Recycling elongation stages

In recycling stages, one inner primer synthesises at the place of loop-stem structure, and initiates the replacement of LAMP, producing as much as 10^9 copies of target gene in less than 60min with several cauliflower-like structure.

Step1. Starting material synthesis and cycling amplification stages



Step2. Recycling elongation stages

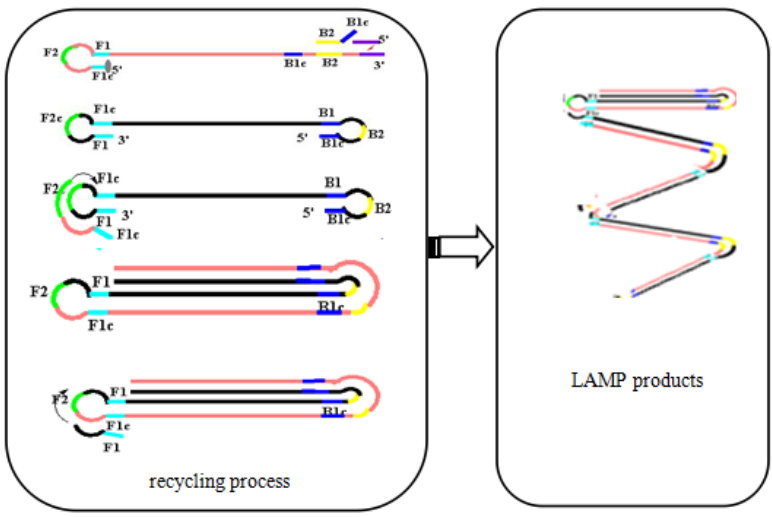


Figure2. Illustration of basic principle for LFD.

Schematic illustration of the experimental procedures for LFD detection of LAMP amplicons

The specific hybridization product of FITC-labeled DNA probe and LAMP amplicons labeled with biotin is combined with Fluorescein isothiocyanate antibodies labeled colloidal gold. Then the ternary complex could be visualized trapped at the LFD strip test line, while the binary complex of free FITC-labeled DNA probe and Fluorescein isothiocyanate antibodies labeled colloidal gold would be trapped at the control line.

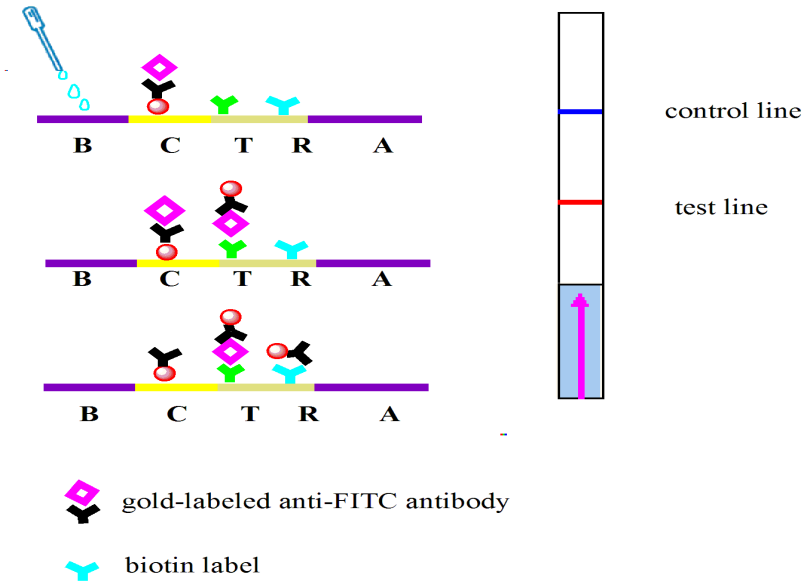


Figure 3. **Illustration of FRET-LAMP**

The FRET probes designed to target loop region of the LAMP amplicons were added to conventional LAMP reagent, and the fluorescently labeled Oligo DNA probes hybridized to the LAMP product, detected easily by naked eye on a conventional UV illuminator. If the amplification setting is connected to a computer with the appropriate software, the results are obtained in real-time as shown in coordinate axis. The fluorescence units are shown on the Y-axis and the time to amplification on the xaxis.

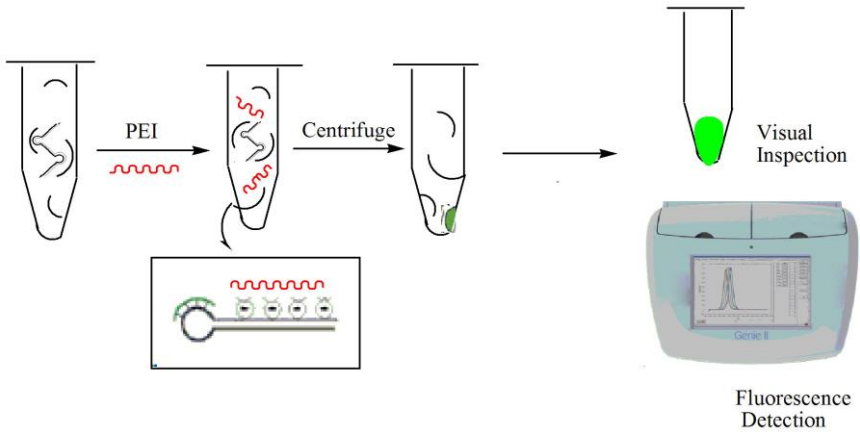
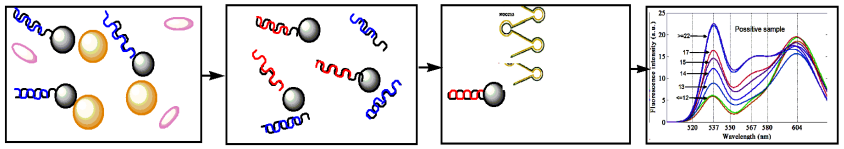


Figure 4 Illustration of Magnetic beads based micro-fluidic

a. The cell was dissolved and the target DNA mixed with the samples was specifically recognized and hybridized onto the surface of the magnetic beads conjugated with specific probe.

b. Nucleic acid amplification of target genes using LAMP conjugated with spectrophotometer and give a semi-quantitative result as shown in coordinate axis.



cell lysis | DNA attached with magnetic beads | LAMP process | Spectrophotometric analysis