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High flux isothermal assays on pathogenic, virulent and toxic genetics from various pathogens

Yanmei Li[a,1], Caiying Bai[b,1], Ling Yang[c], Jie Fu[a], Muxia Yan[a], Dingqiang Chen[c,*], Li Zhang[a,**]

[a] Department of Haematology, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou 510623, China
[b] Guangdong Women and Children Hospital, Guagzhou 510010, China
[c] Department of Laboratory Medicine, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou Medical University, Guangzhou 510120, China

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ABSTRACT

Toxins, encoding by virulence factors, are significant cause of food-borne illnesses and death in the worldwide. Loop-mediated isothermal amplification (LAMP) is one of the widely used methodologies because of the high sensitivity, specificity and rapidity. Nowadays, LAMP has been regarded as an innovative gene amplification technology and emerged as an alternative to PCR-based methodologies in identification of the pathogenic virulent and toxic genetics. The high sensitivity of LAMP enables detection of the pathogens in sample materials even without time consuming and sample preparation. Therefore, we review the typical characteristics of LAMP assay, recent advance in detection of virulence factors and the application of LAMP assay on detection of four commonly virulence factors. As concluded, with the advantages of rapidity, simplicity, sensitivity, specificity and robustness, LAMP is capable of identification the virulence factors. Moreover, the main purpose of this review is to provide theory support for the application of LAMP assay on the virulence factors identification.

1. Introduction

Toxin are small, toxic chemical products formed as secondary metabolites that can contaminate a variety of foodstuffs like fishes, milk and fruits, and it is a major culprit for some adverse reaction [1–4]. Therefore, it poses a potential threat to human and animal health through food chain because of their teratogenicity, mutagenicity, and carcinogenicity [5–8]. The toxins usually encoded by virulent factors or genes, resulting in the possibility to detect the factors by nucleic amplification. Over the past years, scientists have made great efforts on developing a rapid detection method for toxins in foodstuff or clinical isolates [9,10]. However, highly sensitive and selective traditional detection methods, such as liquid and gas chromatography combined with mass spectrometry, are time consuming, expensive and required highly personnel, which is not meet the requirement of "point-of-need" testing and real time food monitoring [11]. There is a fast-increasing and urgent demand for high-performance device to monitor contaminant in complex foods. Simultaneously, there are many people mistakenly believed that the bacteria can be regarded as pathogens once it were isolated from the samples. For example, it dose not mean that the isolated strains is the pathogenic bacteria even though *Escherichia coli* isolated from diarrheal patients [12–14]. In order to accurately and rapidly diagnose infectious diseases cause by toxin-producing bacteria, it is necessary to identify whether the isolates produce related toxins, colonization factors or genes encoding by virulence factors. Nowadays, many methods are available for the detection of toxins, and biological-based method is particularly important for the detection virulence factors of unknown properties [15]. However, the method requires the animal or tissue culture device, and a complicated operation, limiting its application.

With the development of molecular biology, many new molecular diagnostic technologies have been developed. A number of polymerase chain reaction (PCR) and real-time quantitative PCR (RT-qPCR) based assays have been proposed and employed for rapid detection of virulence factors [16,17]. However, obviously disadvantages of PCR (time consumption for post determination, high risk of cross contamination and low detection limit levels) and real-time PCR (requirement for trained personnel, operating space, expensive equipment and reagents) posed significant obstacles for their broad application [18–21]. In 2000, Notomi et al. reported a novel nucleic acid amplification method, designed loop-mediated isothermal amplification (LAMP) [22]. Compared with PCR, LAMP has been regarded as an innovative gene amplification technology and emerged as an alternative to PCR-based methodologies in both clinical laboratory and food safety testing. In terms of results

* Corresponding author.
** Corresponding author.
E-mail addresses: jyksys@126.com (D. Chen), zhangligz06@163.com (L. Zhang).
[1] These authors contribute equally to this work.

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determination, we could judge the positive reaction through the color change of the reaction tube rather than agarose gel electrophoresis, saving nearly 40 min [23–25]. And the high sensitivity of LAMP enables detection of the pathogens in the samples even without time consuming. Thus, LAMP technology has a promising prospect in clinical diagnosis.

Nowadays, LAMP has been applied to detection and identification on pathogens from microbial diseases, as it showed significant advantage in high sensitivity, specificity and rapidity. Moreover, it was also used to confirm whether the bacteria carries the virulence factors. Generally speaking, LAMP has been gradually developed into a mature and reliable assay of molecular biology diagnosis, and it has great potential for the study of local epidemics in endemic countries and regions because of its outstanding advantages [26–29]. Therefore, we will discuss the recent developments of LAMP application on the detection of pathogenic virulent and toxic genetics.

2. Characteristics of LAMP

LAMP, characterized by the nucleic amplification under isothermal conditions, and the results could be judged through the color change by simply adding fluorescent dye SYBR Green I or calcein [30]. Moreover, LAMP products can be detected indirectly by the turbidity that arises due to the formation of insoluble magnesium pyrophosphate. A large amount of pyrophosphate ions is produced by product, yielding a white precipitate of magnesium pyrophosphate in the reaction mixture. As the increased turbidity of the reaction mixture caused by the production of precipitate correlates with the amount of DNA synthesized, monitoring of the LAMP reaction can be achieved by real-time measurement of turbidity. This method relies on auto-cycling strand displacement DNA synthesis performed with the *Bst* DNA polymerase large fragment with a set of two specially designed inner and two outer primers. With an additional one or two loop primers (LF primer and LB primer), the LAMP assay rapidly amplified the target gene within 30 min, requiring only a laboratory water bath for the reaction to occur [31–34]. Over the past decades, LAMP has been combined with other methodologies for different purpose. For example, LAMP has been combined with most probable number (MPN) assay, for enumeration the number of isolates in detail. In addition, LAMP based methodologies combined with ethidium monoazide (EMA) or propidium monoazide (PMA), which intercalates covalently into the DNA, have been applied to detection and identification of foodborne pathogens in viable but non-culturable (VBNC) state from dead cells.

2.1. Current methodologies adaption for detection toxins

Traditional methods for toxin analysis in food include mammalian bioassays, chromatography-based methods and ELISA [35–39]. It brings their own disadvantages in areas such as ethical issues, lack of portability, time consumption and cost-effectiveness. The quintessential method of toxin analysis should be rapid, portable and cost-effective and be able to match the established methods in areas such as sensitivity and selectivity. Biosensor-based methods are now able to fulfil these requirements and also compete with traditional techniques. A broad range of biosensing platforms have been developed and are reported in the literature, utilizing a variety of transducers, sensing platforms, recognition elements and assay formats. However, most biosensor research were in the laboratory stage as the food samples often present complex matrices which have influence on the system. Therefore, nucleic acid detection is the most reliable method.

3. Application of LAMP on detecting virulence gene

3.1. Enterotoxin

Enterotoxins, produced by *Staphylococcus aureus* and *E. coli* or other bacteria, are major cause of diarrhea in infants and young children in developing countries [40–43]. If foods containing enterotoxigenic bacteria are stored under inappropriate temperature condition, sufficient bacteria growth to produce toxic levels of enterotoxins can occur. Indeed, enterotoxin in food has caused outbreaks where the incriminated food had already undergone heat treatment. Because staphlococcal enterotoxins (SEs) are heat stable, heat treatment such as cooking and pasteurization, cannot totally inactive them, resulting in the mentioned adverse reaction. The classical SEs are well-recognized and include SEA, SEB, SEC, SED and SEE. Among these, SEA is the most common toxin implicated in *Staphylococcal* food poisoning. On the other hand, enterotoxigentic *Escherichia coli* (ETEC) strains cause diarrhea through the action of heat-labile enterotoxin (LT) or the heat-stable enterotoxin (ST), and may express LT alone, ST alone, or both LT and ST. In order to detection the production of enterotoxin genes, various commercial kits are currently available. In particular, reverse passive latex agglutination (RPLA) assay for the detection of SEA, SEB, SEC and SED is commonly used in Japan and other countries. Molecular genetic methods were also developed. For example, genes, including *LT* and *ST*, which encode enterotoxins, are mostly identified by PCR [44–47].

Four sets of LAMP primer were designed to detect the SEs gene by Hara-Kudo et al. [48]. And detection limits of *sea*, *seb* and *sed* were reported to be 920 CFU/ml, 32 cell/reaction and 38 cells/reaction, respectively. It was recently reported that there is a detection limit of approx. 1000 cell/ml in real-time PCR assay targeting the *sea*, illustrating that the LAMP assay has similar sensitivity to real-time PCR. The LAMP assay was generally more sensitive than the PCR assay at 30 cycles, and it was 10000-fold higher than that of PCR, especially in assay targeting *sed*. Application of LAMP assays were performed on 64 bacterial strains, including 30 carried genes for enterotoxins, of which 11 sea^+, 13 seb^+, 8 s^+, 7 sed^+ isolates. LAMP assay could identically detect and discriminate all strains judged to be enterotoxigenic by PCR. The strains, negative by PCR and RPLA, were also negative in the LAMP assay. Because a rapid and accurate method for the screening and typing of genes for SEs is very important in food hygiene and epidemiology, LAMP assay targeting genes for newly described SEs should be proved useful for the rapid detection of enterotoxin.

The LAMP assay, conducted by Akemi et al. [49], was considered as a successful method for detecting the heat-labile I (LTI) and heat-stable I (STI) enterotoxins genes. For *LTI*, the limits of detection (LOD) of the method conducted with loop primers was found to be 4 CFU/reaction, while that using conventional PCR assay was 40 CFU/reaction. Thus, the sensitivity of LAMP is 10-fold higher than that of conventional PCR. Furthermore, utilization of loop primers allowed for detecting the gene amplification more rapidly. The time required to detect amplification signals decreased 10–20 min. With regard to detection of *STI* gene, LAMP was 10-fold more sensitive than conventional PCR as well. For PCR assay, three or 4 h are required to identify gene amplification, while accelerated LAMP require less than 35min, suggesting that the LAMP detection procedure for *LTI* and *STI* is superior to conventional PCR methods in terms of sensitivity and rapidity. As for specificity, eighty LT and ST non-producing *E. coli* strains were subjected to detection by the aforementioned LAMP assays. The specificity of LAMP assays for the *STI* and *LTI* was 100%, 100%, respectively. However, non-specific amplification in 4 strains was observed on PCR assay. Thus, LAMP has higher specificity when compared with PCR assay. Detection of *LTI* and *STI* from ETEC employing the established LAMP assay allows one-step identification of gene amplification without specialized equipment, and a wider application in clinical use is expected.

3.2. Shiga toxin

The shiga toxin family is made up of 2 main groups, Shiga toxins 1(Stx1) and Shiga toxin 2 (Stx2), produced by *E. coli* O157: H7 and