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Full Length Research Paper

Drawback of loop-mediated isothermal amplification

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Loop-mediated isothermal amplification (LAMP) was a novel nucleic acid amplification method that amplified DNA with high specificity, efficiency and rapidity under isothermal conditions using a set of four specially designed primers and a DNA polymerase with strand displacement activity. In our research, LAMP was developed to detect foodborn *Salmonella* in raw milk, a set of six primers, two outer, two inner and two loop primers, were designed from *Salmonella* genomic DNA targeting invA. Temperature condition for detection of *Salmonella* was optimized to be 61℃. At the same time, LAMP was found to be effective when the template was pure; however, the usefulness of LAMP methods can be limited by the presence of inhibitors in the analysis of raw milk, unlike all the reports about LAMP, the sensitivity of LAMP was lower than that of PCR methods.

Key words: Loop-mediated Isothermal Amplification, LAMP, Foodborn *Salmonella*, raw milk, sensitivity.

INTRODUCTION

Recently, a new technique called loop-mediated isothermal amplification (LAMP) had been developed which can amplify nucleic acids with high specificity, sensitivity and rapidity under isothermal conditions (Notomi et al., 2000). The method was easy to perform (Mori et al., 2001; Nagamine et al., 2002a), which was based on the principle of the reaction performed by a DNA polymerase with strand displacement activity and a set of two specially designed inner primers (FIP and BIP) and two outer primers (F3 and B3). LAMP had high specificity for the target sequence because the target sequence was recognized by six independent sequences (F1c, F2, F3, B1c, B2 and B3) in the initial stage and by four independent sequences (F1c, F2, B1c, and B2) in the later stages of the LAMP reaction. The amplification efficiency of the LAMP method was extremely high because of its no time loss for thermal change, based on its isothermal reaction. Therefore, the LAMP assay had the advantage in specificity, selectivity and rapidity over other nucleic acid amplification methods (Mori et al., 2001). Moreover, Nagamine et al. (2002) had advanced the method by putting forward loop primers (LF and LB) that accelerated the LAMP reaction.

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Salmonella was an aetiologic agent of diarrhoea and a source of many food-related outbreaks (Trafny et al., 2006), detection of *Salmonella* was an important parameter in microbiological analysis to control food safety, many methods had been developed, among which standard culture method was commonly used, but culture method was laborious and time-consuming, requiring nonselective pre-enrichment, selective enrichment, plating on differential agar media, presumptive biochemical identification, and serological confirmation (Španová et al., 2001). LAMP may be a rapid and sensitive method to replace culture method.

In the present study, an assay was developed for detecting foodborn *Salmonella* with invA in raw milk using LAMP, unfortunately, unlike all the reports about LAMP, LAMP method was not superior to PCR method, hereby, and the drawback of LAMP was reported for the first time.

MATERIALS AND METHODS

Bacterial strain and artificially polluted raw milk

Salmonella enterica 13076 (Key Laboratory of Dairy Science, Ministry of Education of China) was stored at –80℃ until use.

Raw milk was subjected to treatment of pasteurization and spread on BS agar to determine that there was no *Salmonella*. Samples of 200 ml raw milk, free of *Salmonella*, were polluted with *Salmonella*, and the concentrations of *Salmonella* were 10^8, 10^7, 10^6 and 10^5 CFU ml^{-1}, respectively.

Table 1. Purity of DNA templates extracted with different methods.

	Read(260)/Read(280)	Read(230)/Read(260)	Abs(260)	Concentration
Method i)	1.8048	0. 5483	1.0169	—
Method ii)	1.5197	1.0986	0.9214	—

DNA preparation

i) DNA from a pure culture of *S. enterica* 13076 was extracted according to the manufacturer's instructions of UNIQ10 Column DNA Extraction Kit (Shanghai Bioengineering Co., Ltd), the DNA template was used for determining the effect of temperature and the effect of DNA purity on LAMP.

ii) Bacteria were split by SDS and protease K, cell debris and polysaccharides were precipitated by cetrimonium bromide (CTAB), total DNA then was precipitated and extracted by isopropyl alcohol (Li et al., 2005), the DNA template was used for determining the effect of DNA purity on LAMP.

iii) Artificially polluted milk was centrifuged at 10 000 rpm for 10 min to obtain bacterial pellet, and then bacterial genomic DNA was prepared by lysis of the bacterial pellet in 100 Al of lysis buffer (20 mM Tris–HCl, pH 8.0, 2 mM EDTA, pH 8.0 and 1.2% Triton X-100) that was boiled for 10 min (Enosawa et al., 2003; Yano et al., 2007). The DNA template was used for comparing sensitivities of LAMP and PCR.

Design of primers

Based on the invA (GenBank Accession No: NC_006905) of *Salmonella enterica subsp. enterica serovar Choleraesuis str. SC-B67*, six primers were designed using PrimerExplorer3, the forward inner primer of invA (FIP) consisted of the complementary sequence (20 nt) of F1, a TTTT linker and F2 (19 nt): 5'-GCGAGGTTTGGCGGCTGATAttttTCGCGACGACAAAATCTGG -3'; the backward inner primer of invA (BIP) consisted of B2 (21 nt), a TTTT linker and the complementary sequence (21 nt) of B1: 5'-AACTTTAGCGCAAGGTGCCTCttttTGCCGGT AAACTACACG-ATGACATG-3'; Primers F3 (20 nt) and B3 (20 nt) for invA were 5'-CTGGGCGTTTTTTTGTCCTG -3' and 5'-GGGAAGGTTAAGGAGGGTGA -3'; loop primers LF (18 nt) and LB (19 nt) for the set of primers were 5'- AGGCTTCGCGTACAGAGG -3' and 5'- CACGTCAGCAAAGCGTACC -3'.

Temperature determination of amplification

LAMP was performed in a total 25-ml reaction mixture containing 0.8 mM each of FIP and BIP, 0.2 mM each of the outer primers, 0.4 mM each of loop primers F or B or F and B, 1.6 mM dNTPs, 1M betaine (Sigma), 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 4 mM $MgSO_4$, 0.1% Triton X-100, 8 units of the Bst DNA polymerase large fragment (New England Biolabs), and the specified amounts of double stranded target DNA (Nagamine et al., 2001; Nagamine et al., 2002; Fukuta et al., 2003; Gunimaladevi et al., 2004). The reaction temperature (57°, 59°, 61°, 63° and 65°C) was optimized, and LAMP was carried out for 60 min and terminated at 80°C for 10 min (Yeh et al., 2005; Itano et al., 2006). LAMP products were subjected to electrophoresis on a 2.0% agarose gel and visualized under UV light of ChemiDocXRS after ethidium bromide staining (Yano et al., 2007; Shoemaker and Klesius, 2006).

Effect of DNA purity on LAMP

The purity of DNA extracted with methods i) and ii) were determined by UV-Visible Spectrophotometer 100Conc and then the templates were subjected to LAMP reaction. The reaction system and conditions were the same as above.

Detection limit of LAMP and PCR

Detection Limit of Salmonella with invA by LAMP

The DNA extracted from artificially polluted raw milk samples by method iii) were used as templates for LAMP following the conditions described above. The products were subjected to electrophoresis on a 2.0% agarose gel and visualized under UV light of ChemiDocXRS after ethidium bromide staining.

Detection limit of *Salmonella* with invA by PCR

In order to make a comparative analysis of both the protocols (LAMP and PCR), DNA obtained from artificially polluted raw milk samples were simultaneously subjected to LAMP and PCR. The PCR reaction was carried out according to the method described by Malorny et al (2003).

RESULTS

DNA template purity

The purity of DNA template extracted by the method i) was higher than that extracted by the method ii), as indicated in Table 1. There were proteins and sugars in the DNA template extracted by the method ii).

Effect of Temperature on LAMP

The LAMP was carried out using the DNA extracted by method i) as template to determine the optimal temperature. From Figure 1, it can be seen that LAMP products were formed at all temperatures. However, 61°C was considered as the optimal temperature as specificity of the reaction increased at this temperature.

Effect of DNA purity on LAMP

DNA templates extracted by method i) and method ii) were used to determine the effect of DNA purity on LAMP. As indicated in Figure 2, there was no product when using the DNA template extracted by method ii); however, when

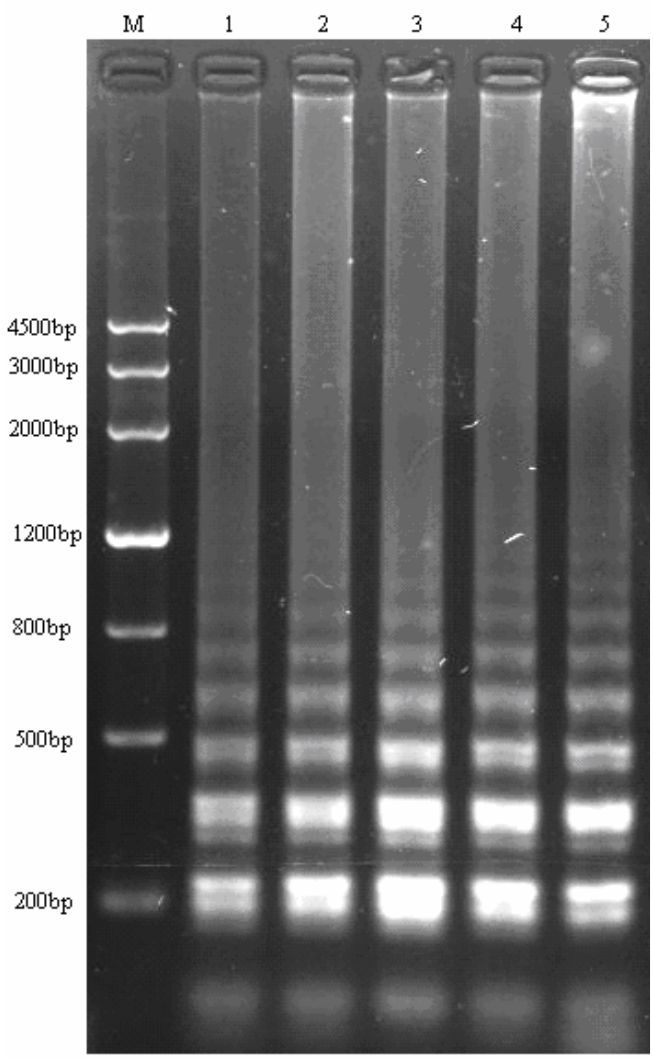

Figure 1: Effect of Temperature on Amount of LAMP Products
M: Marker III; Lanes 1 – 5: LAMP carried out at 57℃, 59℃, 61℃, 63℃ and 65℃, respectively. All the products were electrophoresed on 2% agarose gels and stained with ethidium bromide.

using the DNA template extracted by method i), the result was reverse.

Detection limit of LAMP and PCR

Comparative analysis of sensitivity of *Salmonella* detection by LAMP and PCR was carried out using 10-fold serial dilutions of *Salmonella* DNA. The test was repeated for 20 times by our 5 researchers. Figure 3 shows that LAMP could not detect *Salmonella* even when the DNA template was extracted from the artificially polluted raw milk with 10^8 CFU ml^{-1} *Salmonella*, while PCR could amplify up to 10^6 CFU ml^{-1}. Hence, the detection limit or sensitivity of LAMP was lower than PCR.

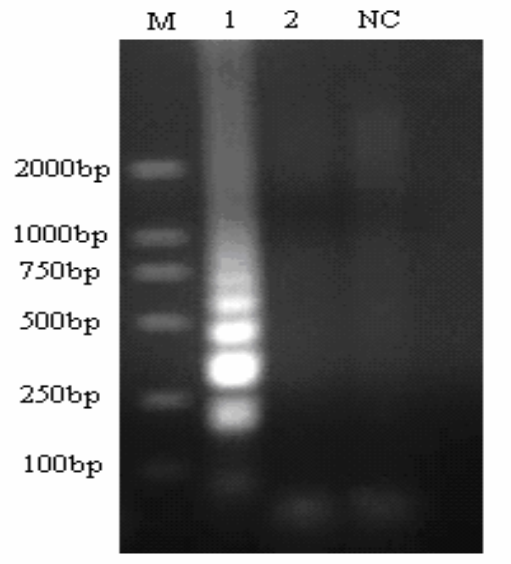

Figure 2. Effect of DNA Purity on LAMP M: Marker DL2000; Lane1: LAMP carried out with the DNA template extracted with method i); Lane2: LAMP carried out with the DNA template extracted with method ii); NC: Negative Control.

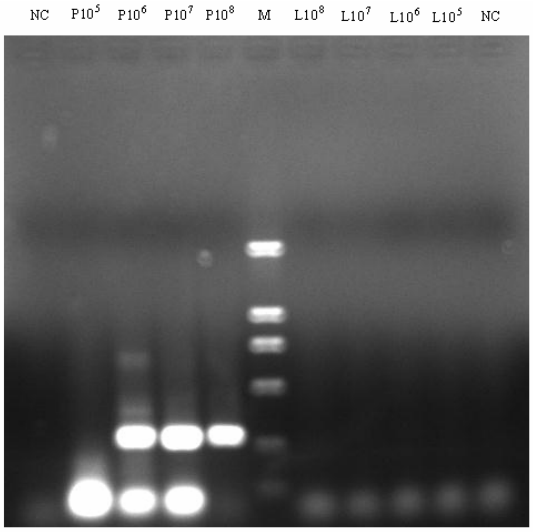

Figure 3. Detection of *Salmonella* DNA by LAMP and PCR from artificially polluted raw milk.
M: Marker DL2000 (2000bp, 1000bp, 750bp, 500bp, 250bp and 100bp); $P10^8$, $P10^7$, $P10^6$ and $P10^5$ were the lanes detected by PCR; $L10^8$, $L10^7$, $L10^6$ and $L10^5$ were the lanes detected by LAMP; NC: Negative Control.

DISCUSSION

Since the development of LAMP, many applications and modifications had been reported, including using the me-

thod to detect foodborn pathogens in food (Wang et al., 2007). Almost in all the reports, the detection limit of LAMP had been found to be higher than that of PCR. Furthermore, in the research of Kaneko et al. (2007), the toleration of LAMP for biological substances was found to be superior to PCR, so they suggested that the DNA extraction step can be omitted in LAMP assay.

Reported DNA preparation methods (Enosawa et al., 2003; Yano et al., 2007) were used to detect foodborn pathogens in raw milk with LAMP in our research. For most foodborn pathogens such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Shigella* and etc, the DNA extraction step can be omitted as suggested by Kaneko et al (2007). However, when detecting *Salmonella* with LAMP and primers as described above, there were plentiful products if the DNA template was pure; in other hand, there was no amplified product if the DNA template was not pure or DNA extraction step was omitted, and the detection limit of *Salmonella* by LAMP was stupendously lower than that of PCR.

The situation may be caused by two reasons: firstly, as reported by Kaneko et al. (2007), a wide range of inhibitors including organic and inorganic substances such as detergents, antibiotics, phenolic compounds, enzymes, polysaccharides, fats, proteins, and salts might inhibit LAMP and PCR reaction; secondly, the primers may have indirect influence on LAMP reaction, because DNA purity did not affect the detection of other foodborn pathogens in raw milk with LAMP. The viewpoints speculated by us need to be further verified.

In a word, further research on stability and sensitivity of LAMP is still in need for generalization, popularization and application of LAMP in clinical diagnosis and food detection fields.

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