Original Article

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Detection methods for milk pathogenic bacteria by loop-mediated isothermal amplification

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Summary **Milk is a common food, which is consumed all over the world. It is an important source of calcium. Meanwhile, it provides abundant protein, minerals and vitamins. However, pathogenic bacteria which exist in milk not only causes nutrition loss, but also produces toxins which may cause diarrhea, food poisoning, and even death. In order to control the microbial level of raw milk and eliminate the contamination of materials, this assay applied loop-mediated isothermal amplification to explore a new way to detect enterotoxigenic *Escherichia coli* (ETEC) in raw milk. The best reaction condition in detecting ETEC from raw milk was confirmed to be: 0.016 μM each of forward outer primer (primer F3) and backward outer primer (primer B3), 0.128 μM each of forward inner primer (primer FIP) and backward inner primer (primer BIP), 0.45 μM deoxy-ribonucleoside triphosphate (dNTPs), 2IU Bst DNA polymerase large fragment and template DNA were incubated at 63°C for 60 min. LAMP was proved to be specific, rapid and sensitive in detecting pathogenic bacteria which exist in milk.**

Keywords: Enterotoxigenic *Escherichia coli* (ETEC), loop-mediated isothermal amplification (LAMP), optimization of reaction condition, rapid detection, raw milk

1. Introduction

Food safety is a worldwide problem (*1*). Enterotoxigenic *Escherichia coli* (ETEC) is responsible for many large-scale foodborne disease outbreaks all over the world (*2,3*). Annually, ETEC is estimated to cause 200 million diarrheal episodes and approximately 380,000 deaths (*4,5*). The pathogenesis of ETEC-induced diarrhea is similar to that of cholera and includes the production of enterotoxins and colonization factors. The clinical symptoms of ETEC infection can range from mild diarrhea to a severe cholera-like syndrome (*6*).

Many methods have been developed to detect ETEC. Monoclonal-antibody method is time-consuming and requires specialized equipment (*7*). Rapid detection method Real-Time quantitative polymerase chain reaction (qPCR) has advantages of rapidity, sensitivity and specificity, but it requires expensive instruments: a qPCR system (*8*). Moreover, the wide range of inhibitors in biological samples(including organic and inorganic substances such as detergents, antibiotics, phenolic compounds, enzymes, polysaccharides, fats, proteins and salts) can inhibit amplification efficiency (*9,10*). It is essential, therefore, that sensitive, specific, rapid, cheap and simple diagnostic methods be developed for detection of ETEC. A novel nucleic acid amplification technology termed loop-mediated isothermal amplification (LAMP) has been developed to amplify nucleic acid rapidly (*11*). LAMP differs from PCR in that four to six primers and a strand-displacing Bst DNA polymerase recognize and amplify the target gene with high specificity at a constant temperature. Since it is isothermal, LAMP can be performed in a simple water bath, other than an expensive temperature cycling device. In addition, the LAMP assay is advantageous over Polymerase Chain Reaction (PCR) in that positive results can be directly detected through visual observation of turbidity changes, so sophisticated electrophoresis apparatus is not essential.

In the present study, extensive standardization of LAMP method was carried out for rapid detection of ETEC in raw milk. The purpose of this study is (*i*) To optimize the LAMP reaction in terms of temperature,

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time and quantities of primers and Bst DNA polymerase fragment; (*ii*) To analyze the specificity and sensitivity of LAMP; and (*iii*) To establish an effective and low-cost method for detecting ETEC from raw milk.

2. Materials and Methods

2.1. *Bacterial strains and culture conditions*

Ten bacterial strains were used in the present study (Table 1). They were cultured for 24-48 h at 37°C in nutrient broth.

2.2. *Artificial contamination of raw milk*

Raw milk was purchased from retail shops in Chengdu City, Sichuan Province, China. The milk was divided into 25-mL test portions. Each test sample (25 mL) was then inoculated with 2 mL of ETEC cultures, resulting in a spiked level of between 10^8 and 10^9 cfu/mL.

2.3. *DNA extraction*

DNA from the pure culture of ten strains used in this study was extracted according to the manufacture's instruction of UNIQ-10 Spin Column Oligo DNA Purification Kit (Shanghai Bioengineering Co., Ltd., Shanghai, China). The DNA extracted was used as template in the later assay determining the optimum reaction conditions and analyzing the specificity of LAMP in detecting ETEC.

The contaminated raw milk was decimally diluted in buffer (20 mM Tris-HCl, pH 8.0; 2 mM EDTA, pH 8.0 and 1.2% Triton X-100). Then the DNA was extracted as follows: First, 1 mL of dilution was centrifuged for 1 min at 10,000× g (Tabletop refrigerated centrifuge, Thermo Fisher Scientific, Chengdu, Sichuan Province, China). After the supernatant was removed, the pellet was suspended in 200 μL of buffer (20 mM Tris-HCl, pH 8.0; 2 mM EDTA, pH 8.0, and 1.2% Triton X-100). Then the tubes were incubated at 56°C for 20 min (Constant temperature water bath, Jinchengguosheng Co.,Ltd, Jintan, Jiangsu Province, China). Finally, after vortex mixing (Essenscien, US) again, the tubes were centrifuged at 10,000× g for 2 min and the supernatant was used as DNA template in the assay comparing sensitivities of LAMP and PCR.

2.4. *LAMP primers*

In the present study, four sets of primers (Synthesized by Shanghai Sangon Biological Engineering Technology And Service Co., Ltd., China) *targeting the heat-labile enterotoxin A subunit and B subunit encoding gene* (Accession number in Genbank: S60731) were designed for the LAMP reaction (Table 2). Each set consisted of four primers: two inner primers (FIP and BIP) and two outer primers (F3 and B3).

2.5. *Optimization of reaction conditions of LAMP*

2.5.1. *Optimum temperature and time*

All the four sets of primers were tested to determine the set of LAMP primers that would give the clearest strips on 2.0% agarose gel(the assay was kept at 60°C for 60 min) (Electrophoresis equipment, Liuyi instrument factory, Beijing, China). Each reaction mixture consisted of 16 μM each of primer F3 and primer B3, 128 μM each of primer FIP and primer BIP, 4 μL of DNA template, 8 IU of Bst DNA polymerase large fragment, 0.45 μM of dNTPs, 5 μL betaine and 1× Thermopol Buffer (New England Biolabs (Beijing, Ltd.) in a total volume of 25 μL. To determine the optimum time and temperature for the reaction, the amplifications were carried out for different time periods (15 min, 30 min, 45 min, 60 min, and 75 min at 63°C) and temperatures (55, 58, 60, 63, and 65°C for 60 min). The reaction was terminated for 5 min at 80°C. LAMP products were subjected to electrophoresis on a 2.0% agarose gel and observed under UV light after staining with ethidium bromide (Gel imager, Bio-rad laboratories, Milan, Italy).

2.5.2. *Optimum quantities of reagents*

Optimum quantities of primers and Bst DNA polymerase large fragment to be added were also standardized at 60°C for 60 min. The following changes were attempted

Table 1. Bacterial strains used in the present study and their sources

No	Strain	Source
1	Enterotoxigenic *E.coli*: 44247	CMCC
2	Enteroinvasive *E.coli* ATCC44338	National vaccine and serum institute, PRC
3	*Proteus vulgaris* 1.1527	CGMCC
4	*Staphylococcus aureus subsp. aureus* 1.8721	CGMCC
5	*Shigella flexneri* 1.10599	CGMCC
6	*Salmonella enteritidis* 50040	CMCC
7	*Bacillus subtilis subsp. subtilis* 1.4255	CGMCC
8	*Sporosarcina sp.*1.192	CGMCC
9	*Bacillus sphaericus*	Microbiological Culture Collection Center of Xihua University
10	*Enterobacter aerogenes* 1.2571	CGMCC

CGMCC, China General Microbiological Culture Collection Center; CMCC, National Center for Medical Culture Collections.

Table 2. LAMP primers used in this study to detect ETEC by targeting *enterotoxin-encoding gene* (Accession Number in Genbank: S60731)

Primer name	Sequence
Primer set1-F3	5'-AGCGGCGCAACATTTCAG-3'
Primer set1-B3	5'-ATCAATTTTGGTCTCGGT-3'
Primer set1-F1P	5'-GCCATTGAAAGGATGAAGG-GATATGTGATTCTTAATGTG-3'
Primer set1-B1P	5'-ATATCTGAGGGTTTTTTT-CGAAGTCCCGGGCAGTCAAC-3'
Primer set2-F3	5'-CTATGTGCATACGGAGCT- 3′
Primer set2-B3	5'-CTCGGTAGATATGTGATTC- 3′
Primer set2-F1P	5'-CTTGTCATTTCG–GTCTATTACAGAACTATGTTCGGAATATAGCAAC- 3′
Primer set2-B1P	5'-CGAAGTCCCGGGC–CCTTCATCCTTTCAATGGCTTTTTTTTGGGAG- 3′
Primer set3-F3	5′-CCATTATATGCAAATGGCGA- 3′
Primer set3-B3	5′-GCTAAGTGAGCACTTCTCAA- 3′
Primer set3-F1P	5′-GGCATAAGACCTCCGGAAC-GAATTC- TTATACCGTGCTGACTCTAGAC- 3′
Primer set3-B1P	5′-TGATCACGCGAGAGGAACACAA-GAGAAGTGGAAACATATCCGTCA- 3′
Primer set4-F3	5′-ATTACATTTAAGAGCGGCGC-3′
Primer set4-B3	5′-GGTTCCTAGCATTAGACATGCTTT-3′
Primer set4-F1P	5′-GTATGGAATAATAAAACCCCTAAAGCAAACTAGTTTTCCA-3′
Primer set4-B1P	5′-TGTCCTTCATCCTTTCAATGGCAGGTCGAAGTCCCGGGCAGTC-3′

in the reaction to optimize the clearness of strips. Optimization of (*i*) amount of primers: the concentrations of primer B3 and primer F3 were increased progressively from 8 μM to 20 μM, *i.e*., 8 μM, 12 μM, 16 μM and 20 μM, and the concentration of primer BIP and primer FIP were increased from 64 μM to 160 μM, *i.e*., 64 μM, 96 μM, 128 μM, and 160 μM; (*ii*) amount of Bst DNA polymerase large fragment-five different amounts, *viz*., 2 IU, 4 IU, 6 IU, 8 IU, and 10 IU were tried.

2.6. *Detection of LAMP products*

The presence/absence of a whitish precipitate was analyzed visually. To confirm the amplification of DNA, 1.0 μL 10^{-2} diluted SYBR GreenI was added to the reaction mixture and the color change was observed. Meanwhile, LAMP products were subjected to electrophoresis on 2.0% agarose gel and observed under UV light after staining with ethidium bromide.

2.7. *Sensitivity of LAMP assay in detecting ETEC compared with PCR*

To compare the sensitivity of LAMP with PCR, 10-fold serial dilutions of the ETEC DNA extracted from raw milk were used as the template to detect the reaction limit. LAMP and PCR products were subjected to electrophoresis on 2.0% agarose gel and observed under UV light after staining with ethidium bromide.

2.8. *Specificity of LAMP assay in detecting ETEC*

All ten bacterial strains in Table 1 were used as templates to determine the specificity of LAMP reaction. The sample without template served as negative control. To exclude the false-positive result, the products of LAMP with DNA from ETEC as the template were digested by restriction endonuclease EcoRI (the reaction mixture consisted of 18 μL of ddH2O, 10 μL of products of LAMP, 2 μL of 10× NEBuffer and 2 μL restriction endonuclease EcoRI (New England Biolabs (Beijing, LTD) and was incubated at 37°C for 3 h). The products were subjected to electrophoresis on 2.0% agarose gel and observed under UV light after staining with ethidium bromide.

3. Results

3.1. *Determination of optimum conditions of LAMP reaction*

In this study, four sets of primers (primer set 1, primer set 2, primer set 3, and primer set 4) were designed targeting the *heat-labile enterotoxin A subunit and B subunit encoding gene* (Accession number in Genbank: S60731) and synthesized. ETEC could only be detected successfully by LAMP with primer set 3 (Figure 1A). The amplification of the target DNA could be observed under UV light. So in the next study, primer set 3 was chosen as the primer. The amplification of the template was successful with primer set 3 when the reactions were carried out at 63 and 65°C as observed by agarose gel electrophoresis, while the reaction at 55, 58, and 60°C couldn't produce any visible strips on gel electrophoresis (Figure 1B). The shortest time required for amplification by primer set 3 was proved to be 60 min (Figure 1C). Besides, the reaction lasted for 75 min (the other conditions *viz*., temperature, amounts of ingredients were consistent with the reaction that lasted for 60 min) didn't provide clearer strips. So it was concluded that a reaction time more than 1 h didn't provide a more reliable result. As a result, 60 min was proved to be the best reaction duration.

3.2. *Optimum quantities of reagents*

In this assay, amplification of the template DNA was induced only when the concentration of primers was

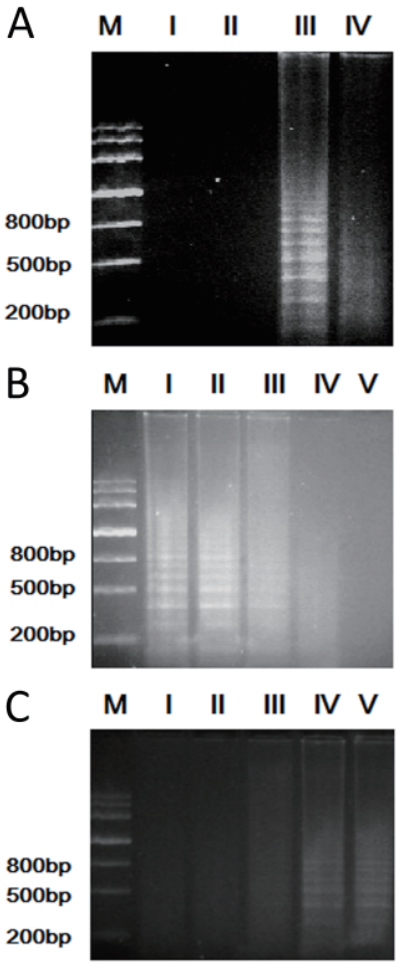

Figure 1. Determination of optimum primer set, temperature and time. (A) LAMP reaction with different primer set. M: DL2000 Plus DNA Marker, I: Primer set 1, II: Primer set 2, III: Primer set 3, IV: Primer set 4. **(B)** LAMP reaction at different temperatures. M: DL2000 Plus DNA Marker, I: 65°C, II: 63°C, III: 60°C, IV: 58°C, V: 55°C. **(C)** LAMP reaction lasted for different times. M: DL2000 Plus DNA Marker, I: 15 min, II: 30 min, III: 45 min, IV: 60 min, V: 75 min.

high enough (primer BIP and primer FIP higher than 128 μM, primer B3 and primer F3 higher than 16 μM) (Figure 2A). More primers led to clearer strips on agrose gel. ETEC could be successfully detected by LAMP with minimal Bst DNA polymerase large fragment (2 IU). It is obvious that reaction systems containing different amounts of Bst DNA polymerase large fragment produced the same results (Figure 2B). So we came to a conclusion that Bst DNA polymerase large fragment isn't the main influential factor.

3.3. *Sensitivity of LAMP assay*

Amplified DNA was observed at dilution 8 in LAMP, which corresponded to 547 cfu/mL, while amplification was observed only up to dilution 5 in PCR (Figure 3). So it was concluded that LAMP assay was 1,000 times more sensitive than PCR reaction in detecting ETEC from raw milk.

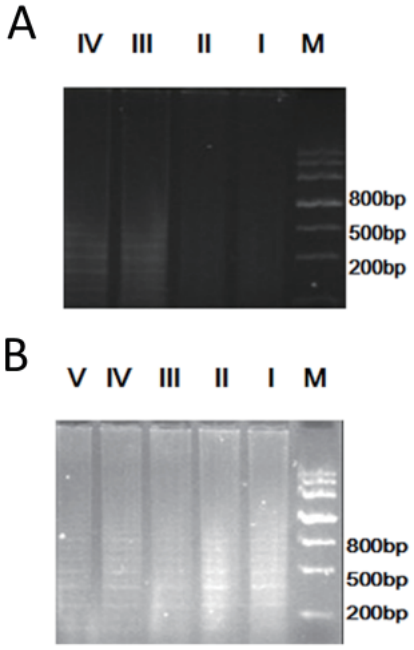

Figure 2. Determination of optimum quantities of reagents. (A) LAMP reaction with different amounts of primers. M: DL2000 Plus DNA Marker. I: primer B3 and primer F3: 8 μM, primer BIP and primer FIP: 64μM. II: primer B3 and primer F3: 12 μM, primer BIP and primer FIP: 96 μM. III: primer B3 and primer F3: 16 μM, primer BIP and primer FIP: 128 μM. IV: primer B3 and primer F3: 20 μM, primer BIP and primer FIP: 160 μM. **(B)** LAMP reaction with different amount of Bst DNA polymerase large fragment. M: DL2000 Plus DNA Marker, I: 2 IU, II: 4 IU, III: 6 IU, IV: 8 IU, V: 10 IU.

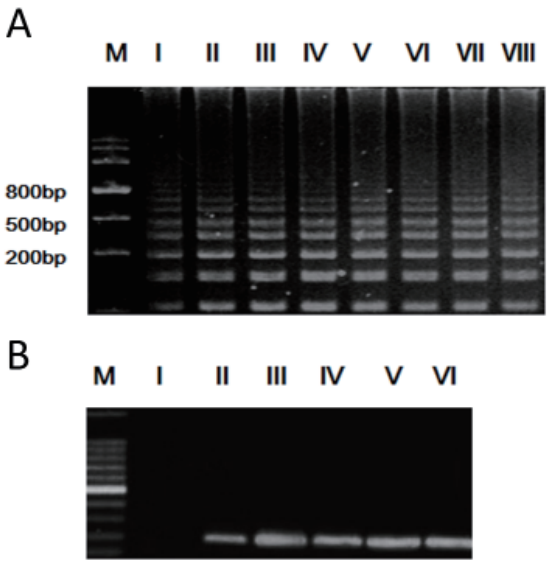

Figure 3. Comparative sensitivities of LAMP and PCR. (A) LAMP products with templates of different dilution rate. M: Marker, I: 10^{-8}, II: 10^{-7}, III: 10^{-6}, IV: 10^{-5}, V: 10^{-4}, VI: 10^{-3}, VII: 10^{-2}, VIII: 10^{-1}. **(B)** PCR products with templates of different dilution rate. I: 10^{-6}, II: 10^{-5}, III: 10^{-4}, IV: 10^{-3}, V: 10^{-2}, VI: 10^{-1}.

3.4. *Sensitivity of LAMP assay*

Among ten bacterial strains studied in this research (one ETEC, one EIEC and eight other bacterial strains), DNA from ETEC was amplified very specifically by

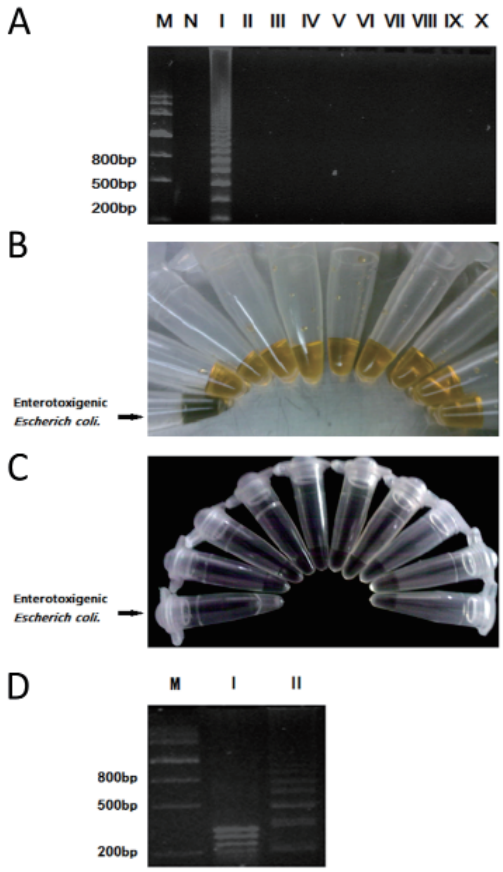

Figure 4. Specificity of the LAMP reaction for detection of ETEC. (A) M: DL2000 Plus DNA Marker. N: Negative control. I: Enterotoxigenic *Escherich coli*. II-X: Strains other than *E.coli*. **(B)** Visual detection using SYBR green 1. **(C)** Detection of whitish precipitate. **(D)** Identification of product of LAMP with template DNA from ETEC. M: DL2000 Plus DNA Marker 1: Fragment digested by restriction endonuclease EcoRI. 2: LAMP product undigested.

the LAMP reaction. The other nine bacterial strains did not provide any precipitate or strips on 2.0% agar gel, nor induce color reaction. After being digested by restriction endonuclease EcoRI, the Lamp product with DNA from ETEC as the template was cut into fragments. The electrophoresis result in Figure 4D proved that the lengths of fragments were consistent with the expected *viz*., 372, 285, 240, and 200 bp. This successfully proved the specificity of LAMP reaction in detecting ETEC.

4. Discussion

LAMP has been widely used as a tool in disease diagnosis (*12,13*), detection of food-borne Pathogenic microorganisms (*14*) and rapid authentication of ingredients (*15*). In the present study, the LAMP method developed was specific for ETEC strain and had no amplified product for 9 non-*E.coli* strains. We reasonably considered that primer set 3 had high specificity for ETEC. Six independent sequences recognized the target gene in the initial stage, and four independent sequences amplified the target sequence in the later stage of LAMP reaction (*16*), while the PCR reaction only had a pair of primers to amplify the target gene. Considering the high sensitivity of LAMP might lead to false-positive strips, we digested the LAMP product with restriction endonuclease EcoRI. The lengths of the fragment digested were consistent with the expected result. This proved the amplification of ETEC DNA, other than background amplification.

The figures presented in this study suggest that the LAMP assay is 1,000 times more sensitive than PCR for detection of ETEC from raw milk. Most studies using the LAMP reaction for detection have found sensitivity to be equal to or 10 times more than conventional PCR/ RT-PCR (*17-20*).

In the present study, it was established that the LAMP reaction for the detection of ETEC works well at both 63 and 65°C (*21*). The present study demonstrated the minimum time for formation of electrophoresis strips at 60 min. This was similar to earlier studies (*21,22*). Following the standardization of temperature and time, optimization of the LAMP assay was also carried out with regard to the effect of the amount of primers and Bst DNA polymerase large fragment on formation of electrophoresis strips on agrose. The electrophoresis strips were found to form with at least 16 μM each of outer primers and 128 μM each of inner primers. In the LAMP reaction, there was no increase of amplification with increasing amounts of Bst DNA polymerase large fragment used. This is an advantage of LAMP assay, which could lower detection cost.

In conclusion, LAMP is a rapid (about 60 min), sensitive (1,000 times more sensitive than conventional PCR assay), cheap (the reaction can be carried out in an isothermal water bath, other than an expensive isothermal-cycling device), convenient (the result could be observed by naked eye, so tedious electrophoresis is not essential), exclusive and inclusive (no false-positive and specific) detection tool for detection of ETEC from raw milk. LAMP technology is friendly for farmers as the detection can be carried out in any simple field laboratory without any professional knowledge. Visualization of the result is convincing to the farmer. It is suitable as a routine diagnostic tool in private clinics and field laboratories.

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