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Development of A Loop-Mediated Isothermal Amplification (LAMP) for the Detection of F5 Fimbriae Gene in Enterotoxigenic *Escherichia coli* (ETEC)

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Abstract The objective of this study was to establish a loop-mediated isothermal amplification (LAMP) method for the detection of F5 fimbriae gene in Enterotoxigenic *Escherichia coli*. A set of four primers were designed based on the conservative sequence of coding F5 fimbriae. Temperature and time condition, specificity test, and sensitivity test were performed with the DNA of *Escherichia coli* (F5+). The results showed that the optimal reaction condition for LAMP was achieved at 61 °C for 45 min in a water bath. Ladder-like products were produced with those F5-positive samples by LAMP, while no product was generated with other negative samples. The assay of LAMP had a detection limit equivalent to 72 cfu/tube, which was more sensitive than PCR (7.2×10^2 cfu/tube). The agreement rate between LAMP and PCR was 100 % in detecting simulation samples. Thus, the LAMP assay may be a new method for rapid detection of F5 fimbriae gene of ETEC.

Introduction

Adhesion fimbria play an important role in causing young animal diarrhea by Enterotoxigenic *Escherichia coli* (ETEC), it enables ETEC to colonize the small intestinal cell surface, and ETEC produce toxins to cause the host diarrhea [1]. The fimbrial adhesions most frequently diagnosed in ETEC strains isolated from piglets with diarrhea are F4(K88), F5(K99), F6(987P), and F41 [14], which have different antigenicity. Thus, vaccines developed from one specific fimbria could not provide protection against an ETEC strain expressing a different fimbria. Therefore, it is necessary to detect fimbriae's type of ETEC-isolated strains and to select an appropriate vaccine prepared with right fimbriae antigen. The type of fimbriae is determined by the genes encoding fimbriae protein, thus detection of the genes encoding fimbriae protein can determine the type that the fimbriae has expressed. F5 fimbriae is one of the most common fimbriae antigen, it was established as a transmissible K antigen with adhesive properties in 1975 [17] and encoded on a 58-megadalton plasmid [8].

Hybridization technique and PCR have been used for detecting F5 fimbriae gene as nucleic acid detection technologies [3, 9, 16], but the concentration of bacteria and purity may make an effect in accuracy and sensitivity with hybridization in practical applications, PCR requires special instruments and equipments, thus extensive application of these technologies are restricted. However, the invention of Loop-mediated isothermal amplification (LAMP) provides new ideas and technologies for establishing a rapid detection method of F5 fimbriae gene.

LAMP is a new technology of nucleic acid amplification reported first in 2000 by Notomi. This method focus on an autocycling strand displacement DNA synthesis performed by the *Bst* DNA polymerase large fragment, which is different from PCR in that four or six primers perform the amplification of the target gene, it is sensitive, specific, and rapid [10]. At present, LAMP has been successfully used to detect many pathogens, such as human malaria parasites, mycobacterium tuberculosis, and Shiga Toxin-Producing *Escherichia coli*, detection of the HCLV against classical swine fever, and so on [6, 11, 18, 22]. The objective of this

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study was to develop a LAMP assay for detecting of F5 fimbriae gene more rapidly, accurately, and easily.

Materials and Methods

Bacterial Strains and Reagents

E. coli C83529 (F5+), C83903 (F4ab+), C83915 (F6+), C83684 (F18ab+), and C83920 (F5+ F41+) were obtained from China Institute of Veterinary Drugs Control. Recombinant *E. coli* TG1 (pMD18T-F41)-contained F41 fimbriae gene was structured in our laboratory; seven simulation samples of *E. coli* strains were prepared by a different Lab in our school, two of the seven strains were F5 fimbria positive and the other five were negative. Bst DNA polymerase was purchased from NEB. Betaine was purchased from Sigma, SYBR Green I was purchased from Xiamen Baiweixin Company (China), and $MgCl_2$ and dNTPs were purchased from Takara. Double-distilled water was used in all experiments. All other reagents were analytical grade.

Primers

Four primers targeting F5 fimbriae protein genes (GenBank Number: M35282) were designed by means of the Primer Explorer V4 (http://primerexplorer.jp/e), including external primers F3 and B3, and internal primers FIP and BIP. A pair of primers P1 and P2 was used for PCR of F5 fimbriae gene [3]. Information regarding the primer names and sequences is provided in Table 1.

DNA Extraction

E.coli C83529 (F5+), C83903 (F4ab+), C83915 (F6+), C83684 (F18ab+), and TG1 (pMD18T-F41) were cultured using LB medium at 37 °C overnight. One milliliter of each *E. coli* culture were transferred into 1.5-ml Eppendorf tube and centrifuged at 12,000×*g*. The supernatants were discarded, the cell pellets were suspended in 500 μL TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA; Sigma-Aldrich), and heated at 95 °C for 10 min in a dry heating block. After centrifugation at 12,000×*g* for 2 min, the supernatants were stored at −20 °C until use as the DNA template for LAMP and PCR.

Table 1 Information on the primers used in this study

Primer name	Sequence(5′ to 3′)
F3	CACGGAACTAAAAAATAATATCGA
B3	GCCCAAGATCTATAGTTGATG
FIP	AGAAGCATTGGTAGTCGCAAAA-ATGAAAAAAACACTGCTAGCTATT
BIP	ACAGGTACTATTAACTTCAATGGCA-CGATTACCATTGACCTCAGG
P1	CTGAAAAAAACACTGCTAGCTATT
P2	CATATAAGTGACTAAGAAGGATGC

Primer Amplification Test

According to the literature [5], the LAMP assay was set up in a total volume of 25 μL consisting of 5 μL of 10 μM FIP and BIP primers, 0.5μL of 10 μM F3 and B3 primers, 2.5 μL of 10 mM dNTPs, 2 μL of 25 mM $MgCl_2$, 2.5 μL of 5 M Betaine, 2.5 μL of 10× Thermo Buffer, 1.5 μL of *Bst* DNA polymerase, and 2 μL of DNA (C83529 (F5+)), and the total volume was made up to 25 μL with deionized water. The reaction was performed in a water bath for 1 h at 61 °C by stopping at 80 °C for 2 min. Then, 3 μL of the LAMP products were separated on a 1.5 % agarose gel. In addition, one microliter of SYBR Green I was added to the reaction tube and the reaction was visualized directly under daylight or under UV light (wavelength: 365 nm).

Optimization of LAMP Reaction Conditions

According to the volume in the literature [5], the LAMP reaction mixtures were incubated at 61, 63, or 65 °C to determine the optimal reaction temperature. When reaction was finished, 3 μL LAMP products were separated on a 1.5 % agarose gel to obtain the desired reaction temperature. Then, the LAMP was performed at the desired reaction temperature for 15, 30, 45, 60, 75, and 90 min to determine the optimal reaction time. The reactions were terminated by heat inactivation at 80 °C for 2 min. The amplified DNA products from the LAMP assays were visualized by 1.5 % agarose gel electrophoresis as above.

Specificity of the LAMP

The specificity of the LAMP assay was tested with the DNA of C83529 (F5+) strain, C83903 (F4ab+) strain, C83915 (F6+) strain, C83684 (F18ab+) strain, and TG1 (pMD18T-F41) strain. The LAMP system was done as described above and the reaction was performed at 63 °C for 45 min. Three microlitre LAMP products were separated on a 1.5 % agarose gel, one microliter of SYBR Green I was added to the reaction tube, and the reaction was monitored directly under daylight or under UV light (wavelength: 365 nm).

Sensitivity Analysis of the LAMP

Template DNA from C83529 (F5+) (3.6×10^8 CFU/ml) was prepared as "DNA Extraction" section described and

diluted for serial tenfold. Optimized LAMP assay and PCR methods [3] were used to detect limits of LAMP and PCR. When reaction was finished, 3 μL of the LAMP products were separated on a 1.5 % agarose gel, one microliter of SYBR Green I was added to the reaction tube and the reaction was visualized directly under daylight or under UV light (wavelength: 365 nm) as above.

Detection of Simulation Samples

Detecting seven simulation samples containing different fimbriae were provided by other staff of this Lab by LAMP and PCR methods [3] respectively. When reaction finished, 3 μL of the LAMP products were separated on a 1.5 % agarose gel, one microliter of SYBR Green I was added to the reaction tube, and the reaction was visualized directly under daylight or under UV light (wavelength: 365 nm) as above.

Repeatability Test

Specificity test, sensitivity test, and detection of simulation samples were conducted in triplicate under the optimized conditions to make sure the development of LAMP is stable.

Results

Primer Amplification Test

The F5 DNA and specific primers targeting F5 fimbriae genes were included in a LAMP performed at 61 °C in a water bath for 45 min. Product of positive reaction with LAMP showed ladder-like pattern on gel electrophoresis, while negative control did not show the characteristic bands (Fig. 1a). The results showed that the primers were effective and specific.

After the addition of SYBR Green I, the product under daylight showed green (Fig. 1b). In contrast, the negative control shows pale orange; else, the product showed green fluorescent under UV light and the negative did not change (Fig. 1c)

Optimization of LAMP Reaction Conditions

The optimal reaction temperature and time of the LAMP were investigated. No significant difference was observed at different temperatures; however, the intensity of DNA at 61 °C showed strongest among all the test temperatures (Fig. 2a). Reactions were then performed at 15, 30, 45, 60, 75, and 90 min at 61 °C. The subsequent results indicated that the DNA products showed the ladder-like pattern literally, when the reaction was performed for 45 min. The optimal reaction condition of the LAMP for F5 fimbriae gene was 61 °C for 45 min (Fig. 2b).

Specificity of the LAMP

The result of detecting by established LAMP were positive only for F5 fimbriae gene, and no positive DNA products of the LAMP assay were observed when these control strains (F4ab, F6, F41, and F18ab) were used as templates (Fig. 3).

Sensitivity Analysis of the LAMP

The results showed that the detection limitation of LAMP was 72 CFU/tube (Fig. 4a–c); in contrast, the PCR has a detection limit of 7.2×10^2 CFU/tube (Fig. 4d). The sensitivity of the LAMP was more sensitive than PCR.

Detection of Simulation Samples

Seven simulation samples were tested by LAMP and PCR assay [2], No. 2 and No. 6 were LAMP-positive samples, as

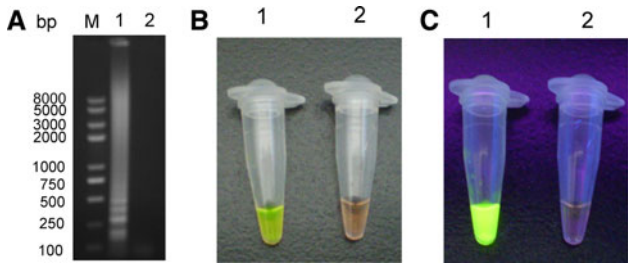

Fig. 1 LAMP detection for F5 fimbriae gene **a** Agarose gel electrophoresis observation of LAMP products; **b** Products of LAMP added with SYBR *Green* I under daylight; **c** Products of LAMP added with SYBR *Green* I under UV lamp (wavelength: 365 nm); *M* DL2000 plus II; *1* positive reaction (with target DNA); *2* Negative reaction (without target DNA) (Color figure online)

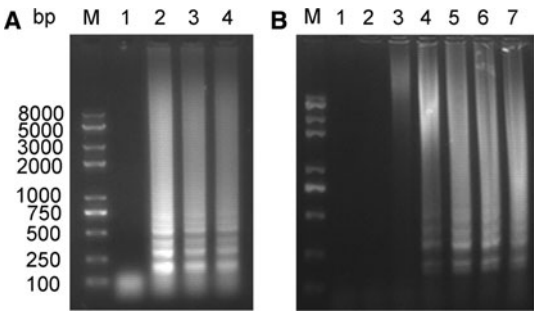

Fig. 2 Amplification of temperature and time optimization **a** Amplification of temperature optimization; **b** Time cost of LAMP reactions; *M* DL2000 plus II; *1* Negative control; **a** *2–4* Products of LAMP at 61, 63, 65 °C, respectively; **b** 2–7: Products of LAMP for 15, 30, 45, 60, 75, 90 min, respectively

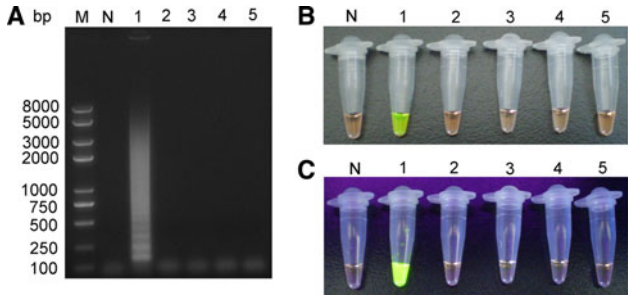

Fig. 3 Specificity test of the LAMP **a** Agarose gel electrophoresis analysis of the LAMP products; **b** Products of LAMP added with SYBR Green I under daylight; **c** Products of LAMP added with SYBR Green I under UV light (wavelength: 365 nm); *M* DL2000 plus II; *N* Negative control; *1–5* The gene of F5, F4ab, F6, F41, F18ab, respectively (Color figure online)

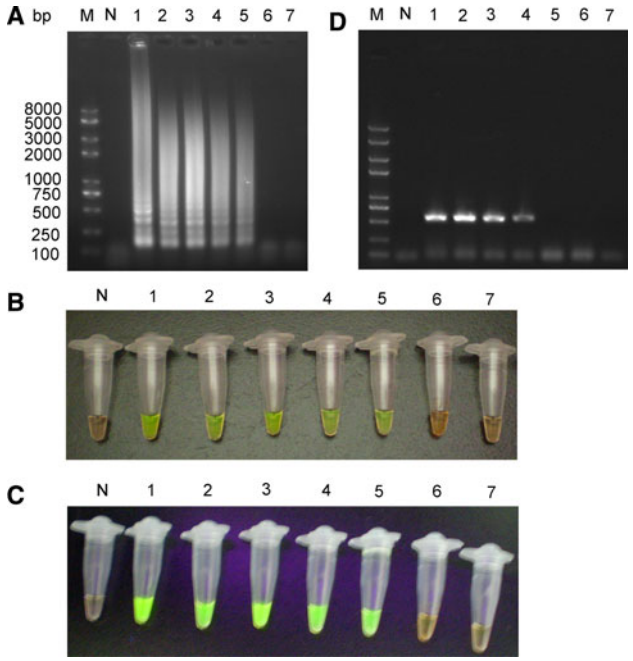

Fig. 4 Sensitivity test of the LAMP **a** Agarose gel electrophoresis analysis of the LAMP products; **b** Products of LAMP added with SYBR Green I under daylight; **c** Products of LAMP added with SYBR Green I under UV lamp (wavelength: 365 nm); **d** Agarose gel electrophoresis analysis of the PCR products; *M* DL2000 plus II; *N* Negative control *1–7* 7.2 × 105, 7.2 × 104, 7.2 × 103, 7.2 × 102, 7.2 × 10, 7.2, 7.2 × 10−1 CFU/tube (Color figure online)

same as PCR results. Test results were consistent with the simulated samples (Fig 5a–d). Simulation samples' strain names and results were shown in Table 2.

Repeatability Test

Specificity test, sensitivity test, and detection of simulation samples were conducted in triplicate under the optimized conditions. It is suggested that the LAMP assay established has good repeatability and stability.

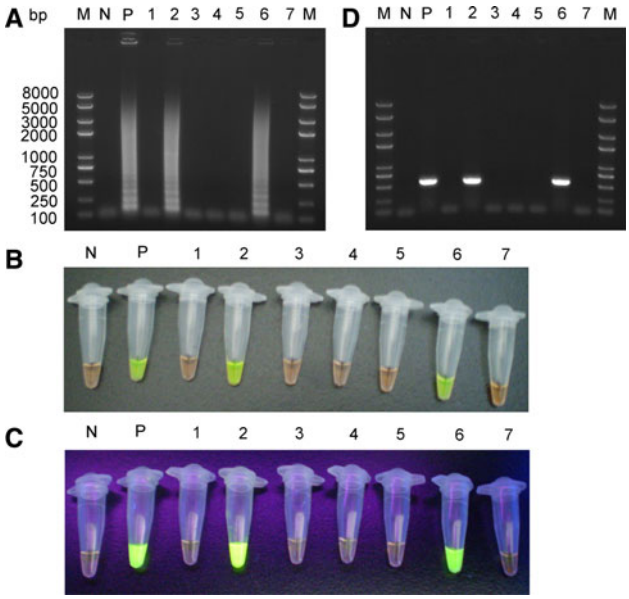

Fig. 5 Detection of LAMP and PCR for simulation samples **a** Agarose gel electrophoresis analysis of the LAMP products; **b** Products of LAMP added with SYBR Green I under daylight; **c** Products of LAMP added with SYBR Green I under UV lamp (wavelength: 365 nm); **d** Agarose gel electrophoresis analysis of the PCR products; *M* DL2000 plus II; *N* Negative control; *P* Positive control (*E.coli* C83529 (F5+)); *1–7* samples (Color figure online)

Table 2 Results of detection of simulation samples

Samples number	Strains name	Results of LAMP	Results of PCR
1	C83684 (F18ab$^+$)	−	−
2	C83529 (F5+)	+	+
3	C83915 (F6$^+$)	−	−
4	TG1 (pMD18T-F41)	−	−
5	C83684 (F18ab$^+$)	−	−
6	C83920 (F5+ F41+)	+	+
7	C83903 (F4ab$^+$)	−	−

Note: "+": simulation samples detected positive with LAMP and PCR; "−": simulation samples detected negative with LAMP and PCR

Discussion

LAMP is a highly specific, sensitive, rapid, and reproducible gene amplification assay. It is easy to perform, can get a lot of specific amplification products at a constant temperature without the need for complex equipment, and the amplification reaction result can be visually determined by turbidity or by adding dye [10]. LAMP has also been used widely for the detection of pathogens [2, 7, 20, 21], but determining results by turbidity requires expensive equipment; thus it is not suitable for small-scale laboratory. In our lab, we added SYBR Green I in the reaction product,

the positive product shows green under daylight; in contrast, the negative control shows pale orange. Else, the product shows green fluorescent under UV light and the negative does not change.

E. coli has a large-scale bacterial genome, about 4 million base pairs, and some sequences are not published in GenBank. Therefore, LAMP primers designed with known genome may result in false positive if they bind on some unknown genome. To guarantee primers' specificity, we appointed upper PCR primer of F5 fimbriae to F2 in FIP of LAMP primers [3], then designed following primers online, thus it reduced workload of detecting primers' specificity. In a LAMP reaction, inner primer F2 in FIP hybridizes to F2c in the target DNA and initiates complementary strand synthesis at first. Outer primer F3 slowly hybridizes to F3c in the target DNA and initiates strand displacement DNA synthesis, releasing a FIP-linked complementary strand, which can form a looped out structure at one end. This single-stranded DNA serves as template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis, leading to the production of a dumb-bell form DNA, which is quickly converted to a stem–loop DNA by self-primed DNA synthesis [10]. So, F2 in FIP and B2 in BIP first combined with target DNA in a reaction, their specificity have a direct effect on the reaction.

Adhesion fimbria is one of the causative agents of ETEC. It mediates ETEC to colonize the small intestinal cell surface and then ETEC produce toxins to cause the host diarrhea; thus, many vaccines about this used fimbriae as the immunogen. Crouch, vaccinated cows with a combined vaccine against rotavirus, coronavirus and *E. coli* F5 (K99). There was a significant increase in the mean specific antibody titer against all three antigens in the serum of the vaccinated animals which was accompanied by increased levels of protective antibodies to rotavirus, coronavirus, and *E. coli* F5 (K99) in their colostrums and milk [4]. Rising [13] experiments showed that sows vaccinated with a vaccine against neonatal *E. coli* diarrhea in piglets containing purified F4ab, F4ac, F5, and F6 fimbriae and detoxified heat-labile toxin (LT) can make an effective protection of piglets against neonatal *E. coli* diarrhea. Rapid detection and identification of fimbriae's type of ETEC strains isolated from animals with diarrhea contribute to select an appropriate vaccine prepared with right fimbriae antigen for improving the immune protective effect, also contribute for epidemiological survey more convenient. So, we can identify the fimbriae type of ETEC that caused young animals' diarrhea, and we can make prevention and treatment against ETEC diarrhea in the more targeted region [9]. ETEC also causes diarrhea in humans, and the major virulence factors of ETEC strains in humans include colonization factor antigens (CFA) and toxins. Some studies suggest that up to 70 % of strains that cause diarrhea in humans express CFA/I, CFA/II, or CFA/IV [12, 15, 19], detection of the fimbriae type is also significant to choose or prepare an appropriate fimbriae vaccine. This study indicates that the LAMP is sensitive, specific, and rapid for detecting F5 fimbriae gene, and the LAMP assay have avoided low specificity, cumbersome operation, special and expensive instruments, and other shortcomings of conventional methods in detecting fimbriae gene. CFA of the ETEC strains in humans may also be detected by LAMP assay.

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