

LAMP 检测无乳链球菌方法的建立及应用

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摘要:以无乳链球菌纤连蛋白 *fbs* 基因为主要研究对象,采取环介导等温扩增技术 (Loop-Mediated Isothermal Amplification, LAMP), 针对 *fbs* 基因的 6 个区域设计 4 条特异性引物,利用一种链置换 DNA 聚合酶 (*Bst* DNA polymerase) 在 63℃ 保温 1 h,通过荧光显色即可完成对无乳链球菌的检测工作。结果显示, LAMP 方法能够特异性检测 *fbs* 基因,其检测灵敏度是常规 PCR 方法的 100 倍,并与实时荧光定量 PCR 方法相当。所建立的针对无乳链球菌 *fbs* 基因的 LAMP 检测方法具有高度的特异性及稳定性,结果可靠,非常适合无乳链球菌的快速检测。

关键词:环介导等温扩增技术;无乳链球菌; *fbsB*; 检测

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Development and Application of Loop-Mediated Isothermal Amplification for Detection of *Streptococcus agalactiae*

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Abstract: To develop a technique for detecting the *fbsB* gene of *Streptococcus agalactiae* using a novel DNA amplification method designated Loop-Mediated Isothermal Amplification (LAMP). The detection assay is based on the loop-mediated isothermal amplification (LAMP) reaction. The *fbsB* gene was amplified by a set of four specially primers that recognize six distinct sequences of the target. The amplification can be obtained in 1 h by incubating all of the reagents in a single tube with *Bst* DNA polymerase at 63℃. Results showed that the developed LAMP assay demonstrated an exceptionally higher than the conventional PCR. This assay results was found to be 100 times more sensitive than the PCR assay, and consistent with the results of real-time PCR. Our results clearly demonstrate that the LAMP-based assay is a sensitive and reliable means for the detection of *Streptococcus agalactiae*.

Key words Loop-mediated isothermal amplification (LAMP); *Streptococcus agalactiae*; *fbsB*; Detection

Streptococcus agalactiae is a highly contagious, obligate bacterium of the bovine mammary gland. Its presence is frequently associated with high somatic cell counts in milk and decreased milk yields^[1,2]. It is considered one of the major causes of economic losses to dairy producers without a control program. The diagnosis of *Streptococcus agalactiae* is typically performed by cultural isolation and serological examinations, but these methods are time consuming and not appropriate for rapid detection in laborator-

ry. Rapid and sensitive laboratory diagnostic methods are required if early treatment for disease is to be initiated. PCR has evolved as one of the most specific and sensitive method for detecting pathogenic microorganisms reported^[3-6]. Recently, Real-time PCR was developed as a powerful tool and its applications of a real-time PCR to microorganisms have been reported^[7-10]. In spite of specificity and sensitivity of PCR and real-time PCR, these methods are not widely used in the detection of

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Streptococcus agalactiae for economic reasons.

A sensitive and specific method for DNA amplification method was developed by Notomi et al. and termed loop-mediated isothermal amplification (LAMP)^[11]. LAMP method relies on auto-cycling strand displacement DNA synthesis by a *Bst* DNA polymerase. This method employs a set of four specifically designed primers that recognize six different sequences on the targeted DNA and amplifies DNA under isothermal conditions. Moreover, LAMP method requires simple incubators, such as a water bath or block heater. In reaction process, a large amount of amplification products are produced with stem-loop DNA structures. Visual inspections of the LAMP amplified product was done by using intercalating dyes like SYBR

Green I, which bind to the double-stranded DNA and produce a green color^[12]. In this study, we have developed a highly sensitive and specific detecting method for *Streptococcus agalactiae*. LAMP method have been designed and compared with PCR and real-time PCR.

1 Materials and methods

1.1 Bacterial strains

A total of 12 pathogens were used to determine the specificity of LAMP detection (Table 1). A *Streptococcus agalactiae* reference strain ATCC 9925 was applied to determine the sensitivity of LAMP, PCR and real-time PCR assay.

Tab.1 Bacterial strains used in this study and results for PCR, real-time PCR and LAMP

Species	Strain No.	Detection of <i>fbxB</i> by		
		PCR	Real-time PCR	LAMP
<i>Streptococcus agalactiae</i>	ATCC 9925	+	+	+
(positive control)	ATCC 624	+	+	+
<i>Staphylococcus aureus</i>	ATCC 6538	-	-	-
<i>Streptococcus bovis</i>	ATCC 9809	-	-	-
<i>Streptococcus dysgalactiae</i>	ATCC 9926	-	-	-
<i>Streptococcus lactis</i>	ATCC 7963	-	-	-
<i>Escherichia coli</i>	ATCC 11229	-	-	-
<i>Bacillus cereus</i>	ATCC 14579	-	-	-
<i>Listeria innocua</i>	ATCC 33090	-	-	-
<i>Pseudomonas aeruginosa</i>	ATCC 27853	-	-	-
<i>Salmonella enterica</i> sub sp.	ATCC 13076	-	-	-
<i>Bacillus subtilis</i> sub sp.	ATCC 6051	-	-	-

Note : + . Positive ; - . Negative.

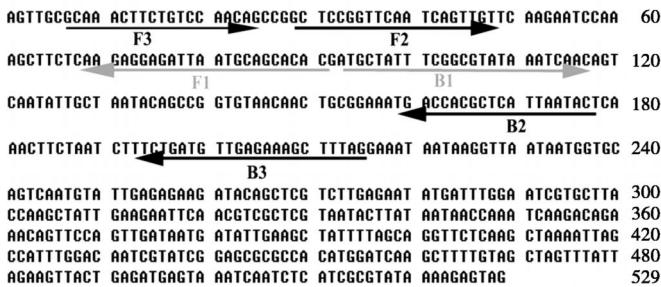
1.2 DNA extraction and preparation

DNA was extracted from an overnight culture of the organism by boiling for 10 min followed by centrifugation at 14 000 g for 3 min. One microliters of the supernatant was used as the DNA template^[13]. For the sensitivity test, an overnight culture of *Streptococcus agalactiae* ATCC 9925 was diluted with sterile water, ranging from 10¹ to 10⁷ CFU/ mL. DNA was extracted as described above. A

negative control was performed using sterile water instead of the DNA template.

1.3 Oligonucleotide primers

Primer design for the LAMP method is illustrated in Fig. 1. A set of four primers comprising two outer and two inner primers was designed. The two outer primers are designated the forward outer primer (F3) and the backward outer primer (B3). The two inner primers are designated



To find specific nucleotide sequences of *Streptococcus agalactiae*, a multiple alignment was determined with analyses of various *fbxB* sequences (YP 329577, NP 687847, NP 735300, from GeneBank data base). The sequences are YP 329577.

Fig.1 Nucleotide sequences of targets for primers in the LAMP assay on *Streptococcus agalactiae* fbsB

the forward inner primer (FIP) and the backward inner primer (BIP). The specific primers for *Streptococcus agalactiae* were designated against the fibronectin-binding protein gene sequences (Gene Bank accession numbers:

YP 329577). The outer primers (F3 and B3) were used as the PCR and real-time PCR primer pair. All primers sequences are listed in Tab. 1.

Tab. 2 LAMP Primers

Primer	Sequence (5'-3')
F3	GCAAACTTCTGTCCAACAG
B3	CTAAAGCTTTCTCAACATCAGA
FIP	GTGTGCTGCATTAAATCTCCTCTTTTGTCTCCGGTCAATCAGTT
BIP	TGCTATTTCGGCGTATAAAATCAACATTTTAGTATTAATGAGCGTGGTCA
F2	GCTCCGGTCAATCAGTT
F1c(sequence complementary to F1)	GTGTGCTGCATTAAATCTCCTCTT
B2	AGTATTAATGAGCGTGGTCA
B1c(sequence complementary to B1)	TGCTATTTCGGCGTATAAAATCAACA

1.4 LAMP reaction

The LAMP reaction was carried out on a scale of 25 μ L reaction mixture containing 40 pmol of each inner primer FIP and BIP ,5 pmol each of F3 and B3 ,8 U of the *Bst* DNA polymerase large fragment (New England Biolabs Inc. ,Beverly ,Mass.) ,1.4 mmol/L concentrations of deoxynucleoside triphosphates ,0.8 mol/L Betaine (Sigma-Aldrich Co. Steinheim , Germany) ,20 mmol/L Tris-HCl (pH 8.8) ,10 mmol/L KCl ,10 mmol/L (NH₄)₂SO₄ ,8 mmol/L MgSO₄ ,0.1 % Tween20 ,and 2 μ L template DNA. The mixture was incubated at 63 for 60 min and then heated at 80 for 2 min to terminate the reaction. By observation of the LAMP reaction tube after adding 2 μ L 1 000 \times SYBR Green I Nucleic acid gel stain ,it was noted whether the color turned green ,indicating that the reaction is positive ,and the color remains orange ,indicating that the negative reaction. For controlling visual inspection ,all the amplified products were viewed in a 2 % agarose gel and documented using a gel imaging system.

1.5 Sensitivity of the LAMP assay

The detection limit of the LAMP assay was estimated by testing a ten fold serial dilution of the *Streptococcus agalactiae* (strain No. ATCC 9925) . The result was compared with the detection limit of PCR and real-time PCR.

1.6 PCR assay

PCR was performed in a 10 μ L reaction mixture consisting of 5 μ L of Go Taq[®] Green Master Mix (Promega Corporation ,Madison ,USA) ,0.5 μ mol/L concentrations of each primer ,and 1 μ L of template DNA. PCR was performed with a thermal cycler (Applied Biosystems ,CA , USA) for 35 cycles. Each cycle consisted of 1 min at 95 ,30 s at 55 ,and 1 min at 72 for the *fbxB* gene. Samples (5 μ L) of the PCR amplification products were

subjected to electrophoresis on a 2 % agarose gel.

1.7 Real-time assay

Real-time PCR reactions were performed in a 10- μ L reaction volume containing :5 μ L of Fast SYBR[®] Green Master Mix (Applied Biosystems , CA , USA) , 0.5 μ mol/L concentrations of each primer ,and 1 μ L of template DNA. Real-time PCR reactions were executed in a StepOnePlus[™] Real-Time PCR System (Applied Biosystems ,CA ,USA) with a temperature profile of 95 for 10 min ,followed by 40 cycles at 95 for 3 s and 60 for 30 s. Samples (5 μ L) of the PCR amplification products were subjected to electrophoresis on a 2 % agarose gel.

2 Results and Analysis

The LAMP assay produced ladder-like patterns on the gel LAMP products were detected only with the *fbxB* gene of *Streptococcus agalactiae*. Positive LAMP tube turned green after 2 μ L of 1 000 \times SYBR Green I Nucleic acid gel stain due to the presence of LAMP products , while the negative tube remained orange in color due to the absence of LAMP products (Fig. 3) .

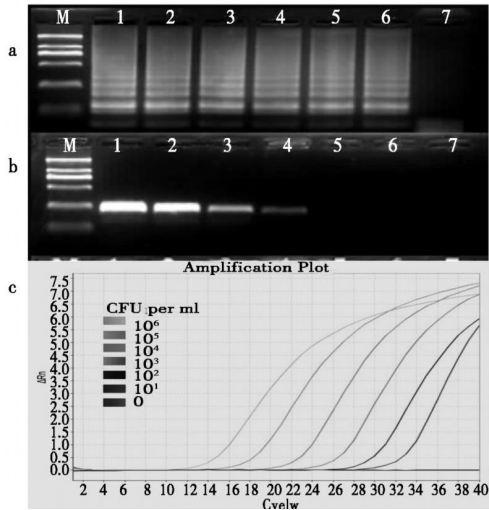
Comparative analysis of sensitivity of detection by LAMP ,PCR and real-time PCR was carried out using 10-fold serial dilutions of *Streptococcus agalactiae*. The detection limit for the LAMP reaction was found to be 10 CFU/ mL for a 60 min reaction as well as in electrophoretic analysis. For the PCR assay ,the detection limit was 10³ CFU/ mL in 90 min. For the real-time PCR assay ,the detection limit was 10 CFU/ mL in 45 min (Tab. 3 and Fig. 2) .

The specificity of LAMP reaction was shown in (Fig. 4) . Reaction products were detected only when *Streptococcus agalactiae* DNA was presented. There were

no amplification products detected with other pathogen.

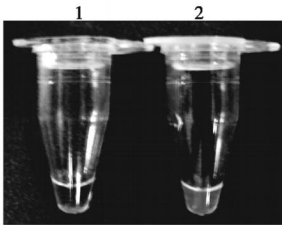
Tab.3 Comparative sensitivities of the LAMP,PCR and real-time PCR methods

Methods	Bacteria number/ (CFU/ mL)						
	10 ⁶	10 ⁵	10 ⁴	10 ³	10 ²	10 ¹	0
PCR	+	+	+	+	-	-	-
Real-time PCR	+	+	+	+	+	+	-
LAMP	+	+	+	+	+	+	-



a. Sensitivity of electrophoretic analysis of LAMP-amplified products ; b. Sensitivity of electrophoretic analysis of PCR products ; c. Sensitivity of real-time PCR for the detection for Streptococcus agalactiae. M. DL2000; 1. 10⁶ CFU/ mL ; 2. 10⁵ CFU/ mL ; 3. 10⁴ CFU/ mL ; 4. 10³ CFU/ mL ; 5. 10²CFU/ mL ; 6. 10 CFU/ mL ; 7.0 CFU/ mL.

Fig. 2 Comparative sensitivity of LAMP, PCR and real-time PCR for detection fbsB gene using serial dilutions of Streptococcus agalactiae



1. Negative LAMP reaction remained orange ;
2. Positive LAMP reaction turned green.

Fig. 3 Visual detection of LAMP products using SYBR Green I stain



M. DL2000; 1. Streptococcus agalactiae (ATCC 9925) ; 2. Streptococcus a- galactiae(ATCC 624) ; 3. Staphylococcus aureus (ATCC 6538) ; 4. Streptococ- cus bovis (ATCC9809) ; 5. Streptococcus dysgalactiae (ATCC9926) ; 6. Strepto- coccus lactis (ATCC7963) ; 7. Escherichia coli (ATCC11229) ; 8. Bacillus cereus (ATCC14579) ; 9. Listeria innocua (ATCC330; 0) ; 10. Pseudomonas aeruginosa (ATCC27853) ; 11. Salmonella enterica subsp. (ATCC113076) ; 12. Bacillus subtilis subsp. (ATCC6071). All the products were elec- trophoresed on a 2 % agarose gels and stained with ethidium bromide.

Fig. 4 Specificity of LAMP primers detecting Streptococcus agalactiae

3 Discussion and conclusion

Until recently ,PCR and real-time PCR have been found to be useful technique for Streptococcus agalacti- ae^[14 - 16] . However ,PCR and real-time PCR are technical- ly demanding and require a relatively long time and need a high-precision thermal cyclcer for a complete detection. In this study ,a novel method called LAMP was used as a detection technique for Streptococcus agalactiae. By con- trast ,the LAMP assay reported here is advantageous owing to its simple operation ,rapid reaction ,and easy detection. Moreover ,LAMP only requires simple reaction equipment ; it can be performed using a regular laboratory bath or heat block that provides a constant temperature of 63 ^[17,18] .

The LAMP assay used for detection is highly sensi- tive ,as it detects 10 CFU/ mL of Streptococcus agalactiae. In the present study ,the assay specifically amplified only Streptococcus agalactiae and no cross-reactivity was ob- served with other bacterial species. Our findings regarding high specificity are in agreement with previous reports of other LAMP methods^[19 - 20] . A 100-fold increase in sensi- tivity was seen in LAMP ,compared to PCR ,where the PCR was 10³ CFU/ mL. We found that the LAMP assay has the same sensitivity as real-time PCR assay.

The LAMP method described in this study represents a very sensitive ,specific ,and rapid alternative mthod to PCR assay for the detection of Streptococcus agalactiae. We recommend that this technique be applied routinely to check milk ,so that bacterium-carrying cow can be found during the early infenction stages.

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