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A Loop-Mediated Isothermal Amplification Method Targeting the *fbsB* Gene for Rapid Detection of *Streptococcus agalactiae*, the Causative Agent of Streptococcosis in Farmed Fish

B. Wang[1,2,3], S.H. Cai[2,3], Y.S. Lu[2,3], X.L. Zhang[2,3], Y.C. Huang[2,3], J.C. Jian[2,3], Z.H. Wu[1,2,3]*

[1] *College of Fisheries, Ocean University of China, Qingdao 266003, China*

[2] *Fisheries College, Guangdong Ocean University, Zhanjiang, China*

[3] *Guangdong Provincial Key Laboratory of Pathogenic Biology and Epidemiology for Aquatic Economic Animals, Zhanjiang, China*

Abstract

The purpose of this study was to develop a loop-mediated isothermal amplification (LAMP) method for rapid, sensitive, and simple detection of *Streptococcus agalactiae* in farmed fish. A set of four primers, two outer and two inner, was designed from the fibrinogen-binding (*fbsB*) proteins gene sequence of *S. agalactiae*, and conditions for LAMP were optimized as incubation of all reagents for 60 min at 64°C. LAMP products were judged by agar gel electrophoresis as well as the naked eye after development by Synergy Brands Green I (SYBR Green I). Sensitivity of the assay was 2.8×10^3 CFU/ml (2.8 CFU per reaction) and no positive amplification was detected for other related bacteria. This LAMP method can be used to detect *S. agalactiae* infection in the laboratory and the field.

* Corresponding author. E-mail: wuzaohe@163.com

Introduction

Streptococcosis is a septicaemic disease caused by *Streptococcus agalactiae* that affects captive and wild fish in freshwater, estuarine, and marine environments. The infection of *S. agalactiae* in epidemic proportions can cause severe economic losses in wild and cultured hybrid (*Oreochromis niloticus* × *O. aureus*) tilapia (Eldar et al., 1994, 1995). Clinical symptoms mainly include evident hemorrhages on the body surface and in internal organs. *Streptococcus agalactiae* is a serious human pathogen, causing a variety of clinical syndromes (Gutekunst et al., 2004).

Streptococcus agalactiae is conventionally detected by isolating the bacteria and physiological-biochemical analysis, which is time-consuming and may delay disease treatment. The pathogen can also be detected by targeting its antigens in infected organs with specific antibodies, but this technique is strongly restricted by cross reactions of antibodies. Thus, a more rapid and sensitive method is required to diagnose streptococcosis in fish at an early stage so as to control the disease at its start.

A novel method termed loop-mediated isothermal amplification (LAMP) amplifies DNA with high specificity, efficiency, and rapidity under isothermal conditions (Notomi et al., 2000). LAMP has wide-ranging applicability in the detection of microorganisms (Fukushima et al., 2002; Shivappa et al., 2008; Yamazaki et al., 2008; Ren et al., 2009). This method employs a DNA polymerase and a set of four specially designed primers that recognize six distinct sequences on the target DNA. LAMP assay is rapid, and its amplification efficiency is equivalent to that of PCR-based methods (Nagamine et al., 2002). More importantly, LAMP is less costly and more practical because all reactions can be developed in an isothermal environment.

Fibrinogen-binding (*fbsB*) proteins are one of the major virulence factors in *S. agalactiae* and could be responsible for mortality in infected hosts (Gutekunst et al., 2004). The *fbsB* protein gene produced by *S. agalactiae* has been cloned and characterized (Gunnar et al., 2005; Pietrocola et al., 2005). Detailed DNA and amino acid sequence analyses of the complete molecule reveal an interesting molecular structure that differs from other gram-positive bacteria. Clarifying and using consensus sequence for *S. agalactiae* strains could lead to more specific and reliable molecular diagnosis of *S. agalactiae* infection. In this study, we developed a rapid and sensitive method using LAMP to detect *S. agalactiae* in fish and evaluated its sensitivity and specificity.

Materials and Methods

Bacterial strains. Sixty-three bacterial strains were used in this study. Some were acquired from diseased fish collected from the coast of the South China Sea, and others were purchased from culture collections (Table 1). To isolate wild-type *Streptococcus* species, colonies appearing on Brain Heart Infusion (BHI; HuanKai Co. Ltd., GuangZhou, China) were chosen and characterized using an Automatic Bacterial Identification System (Biolog Inc, Hayward, CA, USA) and 16s rRNA sequencing (Dorsch et al., 1992). *Streptococcus* spp. were cultured on BHI at 37°C for 16 h; other bacterial cultures were grown on Luria-Bertani (LB) agar (Huankai Co. Ltd.) at 37°C for 20 h.

Table 1. Bacterial strains and sources used in *Streptococcus agalactiae* loop-mediated isothermal amplification (LAMP) experiment.

Species	Strain nos.	Source	No. LAMP-positive/no. strains tested
Streptococcus agalactiae	9925	ATCC	1/1
Streptococcus agalactiae	ZQ0810, ZQ0819, ZQ0910, ZQ0925, ZQ1011, ZQ1020, ZQ1030	Diseased fish, Zhaoqing, China	7/7
Staphylococcus aureus	GZ08, GZ12, GZ36, G48, P08, P12, T65, T78	SCSIO	0/8
Streptococcus bovis	1.6300, 1.6305	IMCAS	0/2
Streptococcus iniae	ZQ0801, ZQ0802, ZQ0902, ZQ0903	Diseased fish, Zhaoqing, China	0/4
Streptococcus lactis	GD01, GD08	GDOU	0/2
Streptococcosis suis	NXY18, NXY56, NXY89, NXY104	GDOU	0/4
Escherichia coli	JM101, DH5α, E07	GDOU	0/3

Table 1 (cont.).

Species	Strain nos.	Source	No. LAMP-positive/no. strains tested
Bacillus cereus	GD89, GD108, GD230, GD289	GDOU	0/4
Pseudomonas aeruginosa	P01, P02, P03, P04, P48, P78, P82, P90	GDOU	0/8
Vibrio alginolyticus	HY9901, NS0701, NS0803, NS0604, MX0702, MX0803,PT0601, PT0604, QH0708, DZ0803, DZ0602	Diseased fish, Zhanjiang, China	0/11
Vibrio harveyi	1.1593	IMCAS	0/1
Vibrio harveyi	Li01, Huang01	GDOU	0/2
Vibrio parahaemolyticus	1.1614, 1.1651, 1.1616	IMCAS	0/3
Aeromonas hydrophila	ZJ04, PT03, GD01	GDOU	0/3

ATCC = American Type Collection, Manassas, VA; GDOU = Guangdong Ocean University, China; IMCAS = Institute of Microbiology of Chinese Academy of Sciences, China; SCSIO = South Sea Institute of Oceanology, Chinese Academy of Science, China

Extraction of DNA. The DNA template was prepared by growing 1 ml *S. agalactiae* in BHI to a late logarithmic phase (16 h post-inoculation) and centrifugation at 12,000 *g* for 5 min to form a pellet. The pellet was resuspended in 200 µl sterile distilled water, boiled for 5 min at 95°C, and centrifuged to collect the supernatant. This supernatant was used for LAMP and PCR.

Design of LAMP primers. A highly specific set of primers (F3, B3, FIP, BIP) was designed according to the *fbsB* gene from *S. agalactiae* (Genbank accession no. YP329577). The primers were designed by PrimerExplorer V4 software program (http://primerexplorer.jp/e/v4_manual/index.html) and custom synthesized by Shanghai Sangon Biologic Engineering & Technology and Service Co. Ltd, China (Table 2).

Table 2. Loop-mediated isothermal amplification (LAMP), common PCR, and real-time PCR primers used in this study.

Primer	Type	Sequence (5′-3′)	Length (nt)
fbs-F3	Forward outer	GCAAACTTCTGTCCAACAG	19
fbs-B3	Backward outer	CTAAAGCTTTCTCAACATCAGA	22
fbs-FIP	Forward inner (F1c-TTTT-F2)	GTGTGCTGCATTAATCTCCTCTTTTTTGCTCCGGTTCAATCAGTT	45 (F1c:22nt, F2:19)
fbs-BIP	Backward inner (B1c-TTTT-B2)	TGCTATTTCGGCGTATAAATCAACATTTTAGTATTAATGAGCGTGGTCA	49 (B1c:25, B2:20)

Optimization of LAMP reaction. LAMP was performed in 25 µl of a mixture containing 2 µl genomic DNA from *S. agalactiae* strain ZQ0810, 40 pmol (each) of primers FIP and BIP, 5 pmol (each) of primers F3 and B3, 1.6 mmol/l dNTP, and 8 U Bst DNA polymerase (New England Biolabs, Ipswich, MA, USA) with the corresponding polymerase buffer. The optimum reaction temperature was determined by incubating the mixture at 58, 60, 62, 64, and 66°C for 60 min. The optimum reaction time was determined by incubating the mixture for 15, 30, 45, and 60 min at 64°C. The reaction was terminated by heating at 80°C for 5 min. LAMP products (3 µl) were detected in 2% agarose with ethidium bromide. For visual detection, results were characterized by the change in color caused by the addition of 1 µl (1:10) SYBR Green I (Molecular Probes Inc, Eugene, OR, USA) to the mixture.

Specificity of LAMP. To evaluate the specificity of detection, LAMP was carried out at 64°C for 60 min with DNA templates from the 63 strains in Table 1. Each DNA sample was examined in triplicate.

Sensitivity of the LAMP assay. A single culture (*S. agalactiae* strain ZQ0810) was inoculated on BHI agar and incubated at 37°C for 16 h. Serial 10-fold dilutions of the culture were prepared in sterile distilled water. To compare the detection sensitivity of LAMP and PCR, DNA extracted from the respective dilutions was subjected to PCR using F3 and B3 primers. The LAMP and PCR sensitivity tests were conducted in triplicate, and detection limits were defined as the last positive dilutions. To enumerate the bacteria,

100 µl aliquots were spread on BHI agar and incubated overnight at 28°C. Colony-forming units (CFU) were counted and CFU/ml was calculated.

PCR assay. PCR was carried out in a total reaction volume of 25 µl containing 2 µl genomic DNA, 2.5 µl buffer solution, 2 µl (10 pmol/l; each) of the F3 and B3 primers, 2 µl dNTP mixture (2.5 mmol of each dNTP), and 2.5 U ExTaq[TM] DNA polymerase (Takara, Shiga, Japan). PCR conditions consisted of an initial denaturation at 94°C for 4 min, 30 cycles of 94°C for 60 s, 55°C for 60 s, and 72°C for 90 s, and a final extension of 72°C for 4 min in a DNA thermal cycler 9700 (Applied Biosystems, Foster City, CA, USA). The amplified products (5 µl) were then analyzed with 1% agarose gel.

Real-time assay. Real-time PCR reactions were performed in 25 µl reaction volume containing 12.5 µl SYBR[®] Premix Ex Taq™ (Takara, Shiga, Japan), 0.5 µmol/l concentrations of F3 and B3 primers, and 1 µl of template DNA in a Real-Time PCR System (Bio-rad iCycler iQ5, USA) with a temperature profile of 95°C for 5 min, followed by 40 cycles at 95°C for 30 s and 60°C 30 s.

Diagnosis of streptococcosis by LAMP. Two groups (n = 5) of hybrid tilapia (*O. niloticus* × *O. aureus*) were held separately in tanks supplied with air-lifted sea water at 25-28°C. Tilapia were artificially infected by intramuscular injection of 0.1 ml bacterial suspension (10^5 CFU/ml) per fish. Control fish received a similar injection of sterile phosphate buffer. At 24 h post injection, liver, kidney, heart, and brain tissues were isolated for DNA extraction using a DNA extraction kit (DNAiso Reagent, Takara, Shiga, Japan) and analyzed by LAMP to detect *S. agalactiae*.

Results

Optimized LAMP conditions. No amplification was detected at 58°C, 60°C, or 66°C (Fig. 1). At 64°C, LAMP products were observed at 45 and 60 min. Thus, optimum conditions for amplification were 60 min at 64°C.

Specificity of LAMP. LAMP specificity was tested using DNA templates extracted from the strains in Table 1. After 60 min at 64°C, using templates of DNA from other enteric bacteria did not produce any amplification (Fig. 2). LAMP products in the positive tubes appeared yellow after addition of SYBR Green I dye, whereas addition of the dye to the negative control tubes did not cause a change in color (Fig 3). The color change was observable to the naked eye.
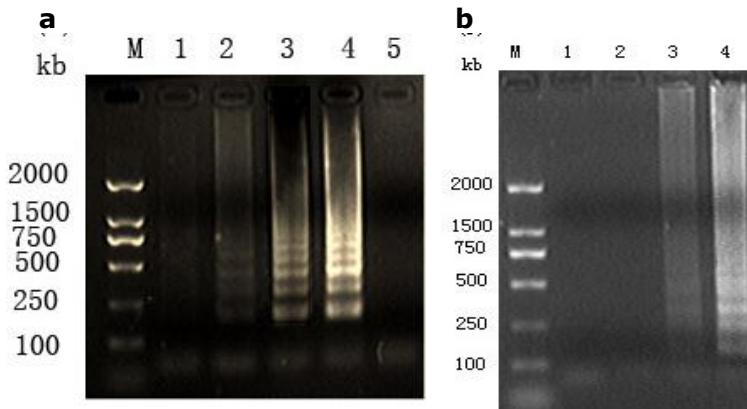

Fig. 1. Determination of optimal (a) temperature (lane M = DL2000 marker, lanes 1-5 = 58, 60, 62, 64, 66°C) and (b) duration (lane M = DL2000 marker, lanes 1-4 = 15, 30, 45, 60 min) for loop-mediated isothermal amplification (LAMP).

Sensitivity of LAMP. The LAMP method detected the *fbsB* protein gene in DNA obtained from 10^3-10^8 CFU of *S. agalactiae*. LAMP reaction detected *S. agalactiae* up to 2.8×10^3 CFU/ml (28 CFU per reaction, Fig. 4), the same sensitivity as real-time PCR. However, PCR detected *S. agalactiae* only up to 2.8×10^4 CFU/ml.

Diagnosis of streptococcosis by LAMP. LAMP was able to amplify *S. agalactiae* from liver, kidney, heart, and brain samples (n = 5) of experimentally infected fish while healthy liver, kidney, heart, and brain samples did not produce amplification when specific primers for *S. agalactiae* detection by LAMP were used (Fig. 5).
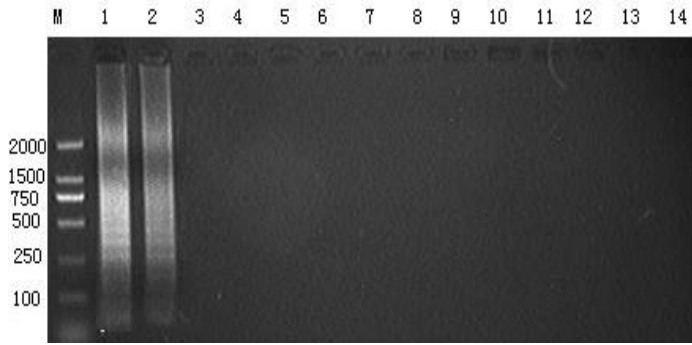
Fig. 2. Identification specificity of the loop-mediated isothermal amplification assay for *Streptococcus agalactiae I:* lane M = DL2000 marker; lanes 1-2 = *S. agalactiae* strains ATCC 9925 and ZQ0810; lane 3 = *Staphylococcus aureus* strain GZ08; lane 4 = *Streptococcus bovis* strain 1.6300; lane 5 = *Streptococcus iniae* strain ZQ0903; lane 6 = *Streptococcus lactis* strain GD01; lane 7 = *Streptococcus suis* strain NXY18; lane 8 *Escherichia coli* strain JM101; lane 9 = *Bacillus cereus* strain GD89; lane 10 = *Pseudomonas aeruginosa* strain P01; lane 11 = *Vibrio alginolyticus* strain HY9901 = NS0701; lane 12 = *Vibrio harveyi* strain 1.1593; lane 13 = *Vibrio parahaemolyticus* strain 1.1614; lane 14 = *Aeromonas hydrophila* strain ZJ04.

Fig. 3. Detection of loop-mediated isothermal amplification (LAMP) products after addition of SYBR Green I. The solution turned yellow in the presence of LAMP amplification products but remained orange in the absence of the amplicon.
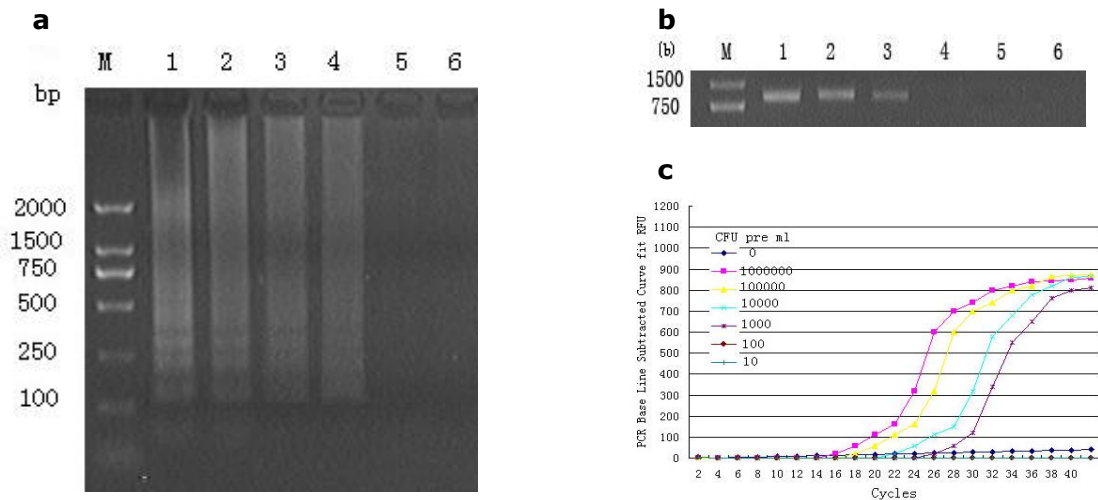

Fig. 4. Sensitivity test for loop-mediate isothermal amplification (LAMP) and PCR using a series of 10-fold dilutions of DNA templates from *Streptococcus agalactiae* (10^{-3} to 10^{-8} dilutions, equivalent to 28,000-0.028 CFU/reaction). (a) agarose gel electrophoresis of LAMP products: lane M = DL2000 marker; lanes 1-6 = reactions using 10^{-3} to 10^{-8} dilutions of *S. agalactiae;* (b) agarose gel electrophoresis of PCR products: lane M = DL2000 marker; lanes 1-6 = reactions using 10^{-3} to 10^{-8} dilutions of *Streptococcus agalactiae;* (c) sensitivity of real-time PCR for detecting *S. agalactiae*.
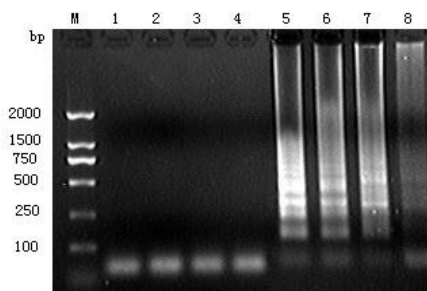

Fig. 5. Detection of *S. agalactiae fbsB* gene in samples from healthy and infected hybrid tilapia: lane M = DL2000 marker; lanes 1-4 = healthy liver, kidney, heart, and brain samples; lane 5-8 = infected liver, kidney, heart, and brain samples. All products were electrophoresed on 2.0% agarose gels.

Discussion

LAMP has been tested to diagnose viruses (Liu et al., 2008), bacteria (Yamazaki et al., 2008), protozoa (Thekisoe et al., 2007), and fungus (Tomlinson et al., 2007). The LAMP primers used in this study were based on *fbsB* sequences, which has some advantages for molecular identification (Courteney et al., 1994). By using the *fbsB* gene for *S. agalactiae*, molecular diagnosis of *S. agalactiae* infection can be more specific and reliable. In addition, the selectivity of the LAMP reaction was extremely high because four primers were used to recognize six distinct regions of the target DNA. The LAMP reaction was positive for all the *S. agalactiae* strains and negative for all other tested species, demonstrating that these primers were specific for identifying *S. agalactiae.* So, LAMP reaction targeting the *fbsB* gene can be used for diagnosis of *S. agalactiae* to the species level.

In addition to high specificity, the LAMP assay for identifying *S. agalactiae* was sufficiently sensitive as it detected 2.8×10^3 CFU *S. agalactiae*/ml while PCR detected only 2.8×10^4 CFU *S. agalactiae*/ml; LAMP was 10-fold more sensitive than PCR, in accordance with results for *Vibrio alginolyticus* (Cai et al., 2010) and *Escherichia coli* (Yano et al., 2007). The greater sensitivity of LAMP could be due to its high amplification efficiency and no time loss for thermal change under isothermal conditions. PCR inhibitors in the samples, which inhibited PCR amplification and reduced PCR efficiency, occurred less in LAMP assay (Mori et al., 2001). This increased sensitivity makes LAMP a better choice than PCR for the diagnosis of *S. agalactiae* in cases where lower concentrations of the bacteria are expected.

The sensitivity of LAMP was almost equal to that of real-time PCR (Wang et al., 2009). Although real-time PCR is superior both in specificity and sensitivity because it allows direct detection of amplified DNA through quantitative measurements, high cost thermocyclers, sequencers, fluorescent probes, dyes, etc., make this method very expensive. So, in cases where economics and practicality of application are constraints, LAMP assay may be more affordable.

We also evaluated the LAMP assay to detect *S. agalactiae* in artificially infected tilapia. Using the LAMP assay, we were able to correctly detect *S. agalactiae* in fish tissues with DNA extracted by kit. Hence, the LAMP assay could be developed into a field test and made available to aquaculturists and producers in an effort to prevent *S. agalactiae.*

In the present work a highly reliable and species-discriminating LAMP-based *S. agalactiae* DNA amplification method was established. This assay may allow more sensitive, specific, and practical detection of *S. agalactiae* than previous detection methods. It provides an important diagnostic tool for the detection of *S. agalactiae* infection in both the laboratory and the field.

Acknowledgements

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