An improved method for detection of *Edwardsiella tarda* by loop-mediated isothermal amplification by targeting the *EsrB* gene*

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Abstract   *Edwardsiella tarda* is a major pathogen in aquatic environments that can cause heavy economic losses. An improved method for quick and accurate detection of *E. tarda* by loop-mediated isothermal amplification (LAMP) with two additional loop primers was developed by targeting the *EsrB* gene (*EsrB*-LAMP). In this method, the Mg^{2+} concentration, reaction temperature, and reaction time were optimized to 8 mmol/L, 61°C, and 40 min, respectively. The detection limit with the *EsrB* gene was as low as 10 copies, which is 100 times more sensitive than that of conventional polymerase chain reaction (PCR). The *EsrB*-LAMP assay was shown more sensitive and rapid than previously reported LAMP assays targeting the hemolysin gene (*hemolysin*-LAMP) for detection of *E. tarda*. The *EsrB*-LAMP was also highly specific to *E. tarda* and had no cross-reaction with 13 other strains of bacteria. The assay can be carried out in a simple heating device and the *EsrB*-LAMP products can be visually detected by adding fluorescent dye to the reaction mixture. Taken together, the improved *EsrB*-LAMP diagnostic protocol has the potential for detection of *E. tarda* from indoor and outdoor samples.

Keyword: *Edwardsiella tarda*; LAMP; detection; *EsrB*

1 INTRODUCTION

Edwardsiella tarda, which was first isolated from a pond-cultured eel (Hoshina, 1962), is a Gram negative bacterium belonging to the Enterobacteriaceae family (Ewing et al., 1965). *E. tarda* can cause serious diseases and even death in many commercially important species of freshwater and marine fish, and thus results in large economic losses to aquaculture worldwide (Thune et al., 1993). Syndromes may include small cutaneous lesions that develop into large necrotic abscesses, ascitic fluid in the abdominal cavity, swollen anus, enlarged kidney, and abscesses on internal organs (Plumb, 1999). In addition to infecting fish, *E. tarda* has a wide host range, and causes diseases in a variety of species including humans (Michael and Abbott, 1993), reptiles, birds (White et al., 1973), and amphibians (Sharma et al.,

1974). To prevent and control this disease, a rapid, sensitive, and reliable technique for early diagnosis is needed.

Traditional methods for diagnosis of bacterial disease are mainly based on phenotypic and serological properties of the pathogen or histological examination (Bernardet et al., 1990; Pazos et al., 1996). These techniques have some disadvantages, such as the need for pathogen isolation, being time consuming, and having low sensitivity. In the past two decades, several PCR-based assays that provide quicker identification than traditional assays have

been applied for the detection of *E. tarda* (Chen and Lai, 1998; Sakai et al., 2007; Lan et al., 2008; Sakai et al., 2009; Castro et al., 2010). However, PCR assays have some intrinsic disadvantages that limit their application, such as requiring the use of PCR instruments, gel electrophoresis, and imaging systems.

Loop-mediated isothermal amplification (LAMP) is a novel method that can detect a few copies of nucleic acids with high sensitivity, specificity, and rapidity under isothermal conditions. The LAMP reaction employs a DNA polymerase with high strand displacement activity and a set of four primers that recognize six distinct sequences on the target DNA (Notomi et al., 2000). Nagamine et al. (2002) developed an accelerating LAMP method that employed two additional loop primers. Specifically, the developed loop F primer (LF) and loop B primer (LB) provide an increased number of starting points for DNA synthesis. *E. tarda* detection by the LAMP method using a set of four primers designed based on the hemolysin gene has been reported by Savan et al. (2004), who also optimized the LAMP reaction conditions for this detection.

Recently, the *E. tarda* type III secretion system (T3SS), which contains 35 open reading frames (ORF), was reported and demonstrated to play an important role in bacterial pathogenicity (Tan et al., 2005; Mo et al., 2007). Therefore, identification of the T3SS gene now makes it possible to identify pathogenic *E. tarda* strains. Here, we report an improved LAMP method for detection of pathogenic strains of *E. tarda* that uses two additional loop primers targeting the *E. tarda* EsrB gene, which encodes a regulator protein of the type III secretion system (Tan et al., 2005; Mo et al., 2007). Additionally, the *EsrB*-LAMP reaction conditions, including the Mg^{2+} concentration, reaction temperature, and reaction time were optimized. In addition, the sensitivity, specificity, and applicability of this assay were discussed.

2 MATERIAL AND METHOD

2.1 Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 1. *E. tarda* strains were cultured in tryptic soy broth (TSB, Beijing Land Bridge Technology, China) at 28°C. *Vibrio splendidus*, *V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus*, *V. fischeri*, *V. anguillarum*, and *Pseudoalteromonas tetraodonis*

were conserved in the laboratory and cultured in 2216E media (5 g/L tryptone, 1 g/L yeast extract, 0.1 g/L FePO₄, 34 g/L NaCl, pH 7.6–7.8) at 28°C. *Staphylococcus aureus*, *Salmonella enteritidi*, *Shigella flexneri*, *Listeria monocytogen*, *Escherichia coli*, and *Bacillus subtills* were cultured in low salt Luria-Bertani medium (LB, 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 8.0) at 28°C. All bacteria had been stored at -80°C in corresponding media containing 15% (v/v) glycerol before use in this study. *E. coli* competent DH5α strain harboring plasmid used in this study were cultured in high salt Luria-Bertani medium (LB, 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0) at 37°C.

2.2 Preparation of bacterial and tissue template DNA

The bacterial genomic DNA was prepared using a TIANamp Bacteria DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions and the direct boiled cells method (Savan et al., 2004), with minor modifications. Briefly, for the direct boiled cells method, 1 mL of log-phase cultured bacterial broth (*E. tarda* 1101) was centrifuged at 5 000×*g* for 3 min to form a pellet. The pellet was then resuspended in 250 μL TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0), boiled for 5 min at 95°C, and centrifuged at 5 000×*g* for 8 min. Next, the supernatant was collected and used for comparison of the detection sensitivity of *EsrB*-LAMP and Hemolysin-LAMP.

Fish tissue total DNA was extracted from 5 mg of the kidneys and guts of infected and healthy *Scophthamus maximus* or *Paralichthys olivaceus* using a QuickGene DNA tissue Kit S (Fujifilm, Japan) according to the manufacturer's instructions.

2.3 Design of primers for *EsrB*-LAMP assay

The *EsrB*-LAMP primers (Table 2) were designed from the *E. tarda* genomic DNA sequence (GenBank accession number CP001135.1) using Primer Explorer version 4 (http://primerexplorer.jp/elamp4.0.0/, accessed on Nov. 12, 2011). A set of four primers (two inner and two outer primers) recognizing six distinct regions on the conservative target-gene sequences (F1c, F2, F3, B1c, B2, and B3) was designed to detect the target genes of *EsrB*. The forward inner primer (FIP) was composed of F1c, a TTTT linker, and an F2 sequence, and the backward inner primer (BIP) consisted of B1c, a TTTT linker, and a B2 sequence. Two additional loop primers (LF and LB) were

Table 1 Bacterial strains used in this study

Strain	Source	Date	Location (China)
E. tarda CD (Wang and Lu, 2009)	Human feces	1991	Jiangsu
E. tarda 072701	*Paralichthy solivaceus*	2007	Shandong
E. tarda 072801	*Scophthamus maximus*	2007	Shandong
E. tarda 080202	*Anguilla japonica*	2007	Guangdong
E. tarda 090101	*Anguilla japonica*	2007	Guangdong
E. tarda 1101 (Yang et al., 2008)	*Scophthamus maximus*	2008	Shandong
E. tarda 050701	*Paralichthy solivaceus*	2008	Shandong
E. tarda 062301	*Scophthamus maximus*	2008	Shandong
E. tarda 081203	*Scophthamus maximus*	2008	Shandong
V. anguillarum MN	*Scophthamus maximus*	2004	Shandong
V. splendidus 082701	*Scophthamus maximus*	2004	Shandong
V. alginolyticus 062307	*Scophthamus maximus*	2006	Shandong
V. harveyi 071802	*Scophthamus maximus*	2006	Shandong
V. fischeri 112505	*Kareius bicoloratus*	2006	Shandong
V. parahaemolyticus 062305	*Scophthamus maximus*	2007	Shandong
P. tetraodonis 031916	*Scophthamus maximus*	2009	Shandong
S. aureus	ATCC 25923		
S. enteritidi	ATCC 13076		
S. flexneri	NICPBP 51572		
L. monocytogen	ATCC 54005		
B. subtills	Maintained in our lab	2006	Shandong
E. coli	Maintained in our lab	2005	Shandong
E. coli DH5α	Takara Co., Ltd	2011	Liaoning

ATCC: abbreviation for American Type Culture Collection; NICPBP: abbreviation for National Institute for the Control of Pharmaceutical and Biological Products, China

Table 2 Primers used for *EsrB*-LAMP

Primers	Primers sequence (5′ to 3′)
F3	ACTCCAGAACCCCCAGG
B3	CCTCGTCCGATATGGCTCA
FIP (F1c+TTTT+F2)	ACCCACCGGCTCAACCTGATTTTGTTGCTCAGCTCCGCC
BIP (B1c+TTTT+B2)	GATGGACAGCAGCTCCGCGATTTTTGACCCTGCGCGAACG
LF	CGCAAGCTGGATGCCCA
LB	TCGGCGACCAGCTTGAGA

designed to accelerate the amplification reaction (Fig.1).

2.4 Optimization of *EsrB*-LAMP assay

The *EsrB*-LAMP reaction was conducted according to the method described by Notomi et al. (2000), with minor modifications. Briefly, the reaction was conducted in a 25 μL reaction mixture containing 1.6 μmol/L each of FIP and BIP, 0.2 μmol/L each of F3 and B3, 0.8 μmol/L each of LF and LB, 1×ThermoPol reaction buffer (20 mmol/L Tris-HCl, 10 mmol/L KCl, 2 mmol/L $MgSO_4$, 10 mmol/L $(NH_4)_2SO_4$, 0.1% Triton X-100, pH 8.8), 6 mmol/L $MgCl_2$ as the additional Mg^{2+}, 1.2 mol/L Betaine, 1.4 mmol/L of each deoxynucleoside triphosphate (dNTP), 1 μL (8U) of Bst DNA polymerase, and
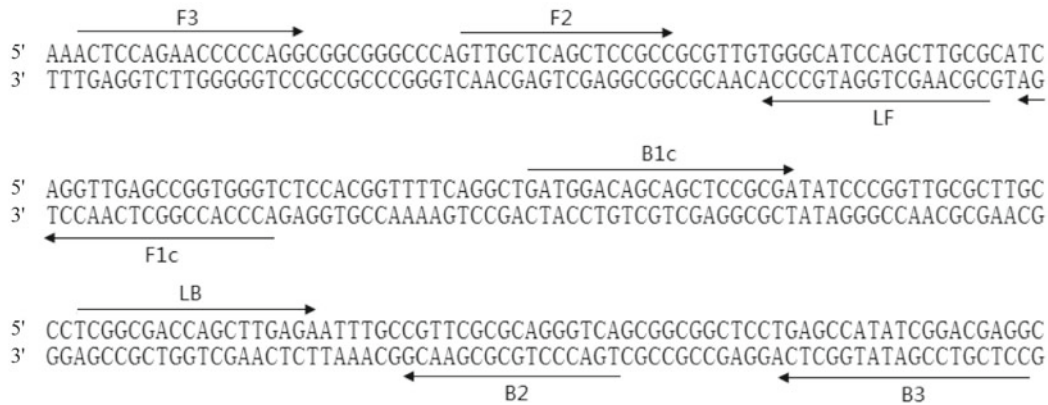
Fig.1 Nucleotide sequence of partial *EsrB* gene of *E. tarda* (GenBank: CP001135.1) used to design primers for LAMP

The nucleotide sequences and the positions used to design the primers are marked with arrows.

0.5 μL of bacterial genomic DNA (10 ng/μL) as the template. After denaturation of the template at 95°C for 5 min, the mixture was incubated at 63°C for 1 h and then inactivated by incubation at 80°C for 5 min. Next, 2 μL of *EsrB*-LAMP products were visualized by 2% agarose gel electrophoresis. To optimize the *EsrB*-LAMP reaction, different total Mg^{2+} concentrations, reaction temperatures, and reaction times were investigated. Gel images were taken using an LAS3000 (Fuji Film, Japan) gel imaging system. The optimal reaction conditions for *EsrB*-LAMP were used in subsequent experiments.

2.5 Detection specificity of the *EsrB*-LAMP assay

The specificity of the *EsrB*-LAMP assay was tested using genomic DNA extracted from nine *E. tarda* strains, six marine *Vibrio* bacteria (*V. splendidus*, *V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus*, *V. fischeri*, and *V. anguillarum*) and *P. tetraodonis*, *S. aureus*, *S. enteritidi*, *S. flexneri*, *L. monocytogen*, *E. coli*, and *B. subtills* as templates. After the reaction, 2 μL of LAMP amplified products were analyzed on a 2% GeneFinder™ (BIOV, Xiamen, China) stained agarose gel.

The amplifying specificity was confirmed by sequencing the *EsrB*-LAMP product. To accomplish this, a LAMP product of approximately 500 bp was used as the template in PCR. The PCR reaction mixture (50 μL) consisted of 1 μL DNA template (50 ng/μL), 25 μL 2×Premix Ex Taq (Ex Taq 1.25 U, 0.4 mmol/L each dNTP, Ex Taq buffer including 4 mmol/L Mg^{2+}) (TaKaRa, Dalian, China), and 1 μL each of FIP and BIP primers of the *EsrB* genes (20 μmol/L). The amplification was performed as

follows: initial denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s and then final extension at 72°C for 10 min. DNA fragments were subsequently purified from agarose gels using a Zymoclean™ Gel DNA Recovery Kit (Zymo, USA), after which they were ligated with the pGM-T EasyVector System (Tiangen, Beijing, China) and cloned into *E. coli* DH5α cells according to the manufacturer's instructions. Next, recombinant plasmids containing DNA inserts were sequenced using the M13 universal primers (forward primer, RV-M: 5′-GAG CGG ATA ACA ATT TCA CAC AGG-3′, reverse primer, M13-47: 5′-CGC CAG GGT TTT CCC AGT CAC GAC-3′). Finally, ClustalX 1.83 was used to analyze and align the sequences with sequences selected from GenBank using the Basic Local Alignment Search Tool (BLAST).

2.6 Detection sensitivity of *EsrB*-LAMP in comparison with conventional PCR assay

To determine the sensitivity of *EsrB*-LAMP and conventional PCR assay, a plasmid containing the *EsrB* gene fragment was constructed. First, the target *EsrB* DNA fragment was amplified from the *E. tarda* 1101 genomic DNA using a pair of primers (forward: 5′-GAT GCC GAT GCC AGA CAA-3′, reverse: 5′-AAA GCC CGC AGC AAA CC-3′) designed from the *E. tarda* genome (GenBank: CP001135.1). The PCR reaction mixture was the same as that described in Section 2.5. PCR was then conducted by subjecting the samples to the following conditions: initial denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for

30 s, and extension at 72°C for 20 s. Next, the PCR products (380 bp) were ligated into the pGM-T EasyVector System (Tiangen, Beijing, China) and cloned into *E. coli* DH5α cells according to the manufacturer's instructions. The sequence of the cloned insert was verified as described in Section 2.5. Ten-fold serial dilutions of the recombinant plasmid (pGM-*EsrB*) were subsequently used as templates to evaluate the detection sensitivity of *EsrB*-LAMP and conventional PCR assays. The results were confirmed by electrophoresis and the sensitivity of two detection protocols was compared. The conventional PCR amplification program was performed as described above. The sensitivity of LAMP was also examined using template DNA obtained from 10^0 to 10^5 CFU of *E. tarda*. This were achieved by conducting a ten-fold serial dilution of 3.0×10^8 CFU *E. tarda* in distilled water by using the direct boiled cells method. After the reaction, 2 μL of LAMP and PCR amplified products were analyzed on 2% and 1.5% GeneFinder™ (BIOV, Xiamen, China) stained agarose gel, respectively.

2.7 The detection speed of *EsrB*-LAMP in comparison with hemolysin-LAMP

To compare the detection speed and sensitivity of *EsrB*-LAMP and hemolysin-LAMP (Savan et al., 2004), a hemolysin-LAMP assay that targeted the hemolysin gene of *E. tarda* was carried out using the reported primers, reaction mixture, and optimal conditions (65°C for 45 min) described by Savan et al. (2004) in parallel with *EsrB*-LAMP. After the reaction, 2 μL of LAMP amplified products were visualized in 2% agarose gel.

2.8 Application of the *EsrB*-LAMP assay to clinical fish samples

In the clinical application of the *EsrB*-LAMP assays for detection of *E. tarda*, 5 ng of DNA templates were extracted from diseased *Scophthatmus maximus* (No. 2009122103-2 and -4) and *Paralichthy solivaceus* (No. 2009102801-2 and -4) intestine tissues, in which the presence of *E. tarda* had been confirmed based on a TaqMan real-time fluorescent PCR assay targeting the 16S rRNA gene. The templates were then used in a PCR assay targeting the *EvpC* gene followed by sequencing analysis (data not shown). In addition, 5 ng of genomic DNA from *E. tarda* 1101 was used as a positive control. Moreover, 5 ng of DNA samples extracted from

healthy *S. maximus* (No 2009122104) and *P. olivaceus* (No. 2009122105) intestine tissues that had been confirmed free of *E. tarda* as described above (data not shown) and distilled water were used as negative controls. The fish samples were obtained from a local farm in Yantai, Shandong Province, China in 2009.

The LAMP assays were visualized by adding 0.5 μL of the fluorescent dye GeneFinderTM (10 000×) to 25 μL reaction mixtures as described by Zhang et al. (2009). Positive or negative results were determined based on different fluorescent colors of the reaction mixtures upon completion of the reaction.

3 RESULT

3.1 Optimization of the reaction conditions for *EsrB*-LAMP

Three crucial components of the *EsrB*-LAMP reaction, Mg^{2+} concentration, reaction temperature, and reaction time, were optimized using *E. tarda* genomic DNA (2.5 ng) as the template. The amplification efficacies with Mg^{2+} concentrations of 2 to 14 mmol/L were tested at 63°C. No amplification product was produced when the total Mg^{2+} concentration was less than 6 mmol/L (2 mmol/L $MgSO_4$ plus 4 mmol/L $MgCl_2$), while the clearest ladder of multiple bands appeared at 8 mmol/L Mg^{2+} (Fig.2a). LAMP products decreased when the total Mg^{2+} concentration increased to 10 and 16 mmol/L; thus, the optimum total Mg^{2+} concentration is 8 mmol/L. The characteristic ladder of multiple bands appeared at temperatures ranging from 55°C to 69°C, but the strongest and clearest ladder was observed at 61°C (Fig.2b). Under the optimum conditions of 8 mmol/L Mg^{2+} at 61°C, the first amplified product was produced in 15 min, but better results were observed after 40 min (Fig.2c). Based on these results, the optimum reaction conditions for *EsrB*-LAMP were 8 mmol/L total Mg^{2+}, 61°C, and 40 min. Therefore, these conditions were used in the subsequent experiments.

3.2 Specificity of the *EsrB*-LAMP assay

DNA samples prepared from *E. tarda* strains, *Vibrio* strains, *P. tetraodonis*, *S. aureus*, *S. enteritidi*, *S. flexneri*, *L. monocytogen*, *E. coli*, and *B. subtills* were used as templates for the *EsrB*-LAMP specificity tests and positive results were only observed upon analysis of reaction mixtures containing the *E. tarda* templates (Fig.3a, b). Moreover, sequence analysis revealed that the sequence of the PCR product
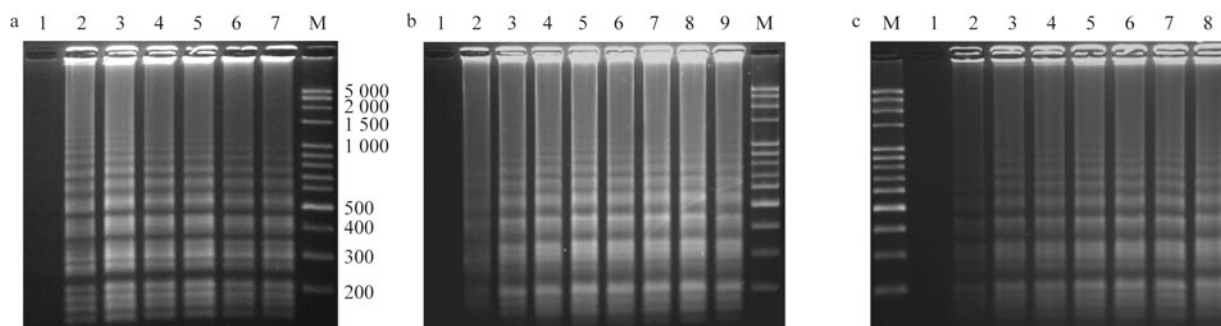
Fig.2 Optimization of *EsrB*-LAMP reaction conditions using the 2.5 ng *E. tarda* 1101 genomic DNA as template

M: 100 bp plus DNA ladder marker (5 000, 3 000, 2 000, 1 500, 1 000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp, Beijing Solarbio, following the same); a. Effect of additional Mg^{2+} concentrations on the LAMP reaction at 63°C. Lanes 1–7: 2, 4, 6, 8, 10, 12 and 14 mmol/L additional Mg^{2+}, respectively; b. Effect of temperature on the LAMP reaction with 8 mmol/L total Mg^{2+}; Lanes 1–9: reaction temperature at 53, 55, 57, 59, 61, 63, 65, 67, and 69°C, respectively; c. Effect of reaction time on the LAMP reaction with 8 mmol/L total Mg^{2+} at 61°C; Lanes 1–8: reactions lasted for 10, 15, 20, 30, 40, 50, 60, and 70 min, respectively.

Fig.3 Specificity test of the *EsrB*-LAMP using genome DNA templates from different bacterial strains

a. Lane 1–9: *E. tarda* strain CD, 072701, 072801, 080202, 090101, 1101, 050701, 062301, and 081203, respectively; Lane 10: *V. alginolyticus* 062307; Lane 11: *V. splendidus* 082701; Lane 12: *V. parahaemolyticus* 062305; Lane 13: *V. fischeri* 112505; Lane 14: *V. harveyi* 071802; Lane 15: *V. anguillarum* MN; Lane 16: Negative control; M: 100 bp plus DNA ladder marker. b. Lane 1: *E. tarda* strain 1101; Lane 2: *P. tetraodonis* 031916; Lane 3: *S. aureus* ATCC 25923; Lane 4: *S. enteritidi* ATCC 13076; Lane 5: *S. flexneri* NICPBP 51572; Lane 6: *L. monocytogen* ATCC 54005; Lane 7: *B. subtills*; Lane 8: *E. coli*; Lane 9: Negative control; M: 100 bp plus DNA ladder marker.

amplified from LAMP products as the template with FIP/BIP primers was identical to the targeted *EsrB* gene of *E. tarda* (GenBank: CP001135.1) (data not shown).

3.3 Comparison of detection sensitivity of *EsrB*-LAMP and conventional PCR

Evaluation of the detection sensitivity of *EsrB*-LAMP using 10-fold serial dilutions from 10^0–10^7 copies of quantified pGM-*EsrB* plasmid DNA revealed that it was 10 copies within 40 min (Fig.4a), while the detection sensitivity of PCR tested using the same templates was 10^3 copies after 35 PCR cycles (Fig.4b). Consistent with the results from the DNA samples prepared from pGM-*EsrB* plasmid DNA, characteristic ladder-like pattern products were detected from the diluted samples of 10 CFU within 40 min (Fig.4c). Thus, the detection minimum of the *EsrB*-LAMP was 10 copies of the targeted gene or 10 CFU *E. tarda*, which was 100 times more sensitive than conventional PCR.

3.4 Comparison of detection speeds of *EsrB*-LAMP and hemolysin-LAMP

The reaction speeds of *EsrB*-LAMP and hemolysin-LAMP were compared using the same template (Fig.5). The first visible banding pattern was produced within 30 min upon *EsrB*-LAMP analysis, whereas the visible banding pattern was produced within 50 min during hemolysin-LAMP analysis. In addition, the degree of brightness and clarity of LAMP amplification products on agarose gel indicated that more amplification products were produced after 50 min of amplification by *EsrB*-LAMP than by hemolysin-LAMP. Thus, the *EsrB*-LAMP was more sensitive and rapid than the hemolysin-LAMP assay, which is in accordance with the results reported by Savan et al. (2004).
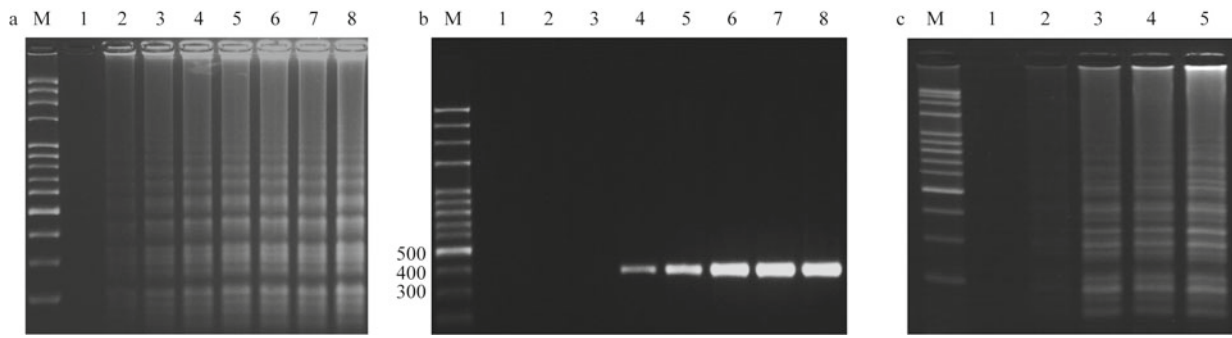
Fig.4 Sensitivities of *EsrB*-LAMP and conventional PCR method for *EsrB* gene

The pGM-*EsrB* plasmid DNA were used as templates in Fig.4a and 4b. DNA template prepared by the boiling method was used in Fig.4c. a, c. LAMP detection within 40 min; b. PCR detection after 35 cycles. Lanes 1–8 in Fig.4a and 4b: 10^0, 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 copies of plasmid DNA, respectively; Lanes 1–5 in Fig.4c: 10^0, 10^1, 10^2, 10^3, and 10^4 CFU of *E. tarda*, respectively; M: 100 bp plus DNA ladder marker.
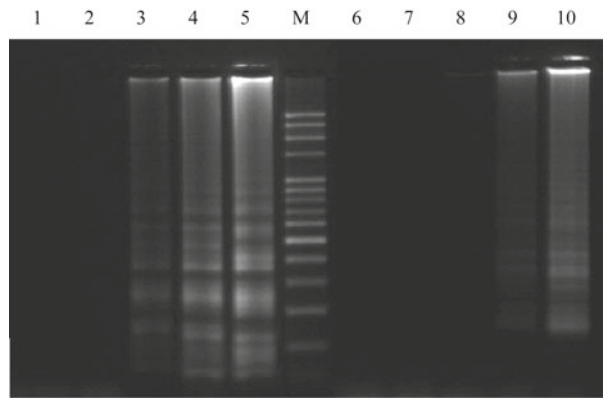

Fig.5 Comparison of *EsrB*-LAMP assay with Hemolysin-LAMP assay using the same *E. tarda* 1101 DNA as template under their optimal reaction conditions, respectively

Lanes 1, 6: negative control in *EsrB*-LAMP and Hemolysin-LAMP within 60 min, respectively; Lanes 2–5: 20, 30, 40, and 50 in *EsrB*-LAMP, respectively; Lanes 7–10: 30, 40, 50, and 60 min in Hemolysin-LAMP, respectively; M: 100 bp plus DNA ladder marker.
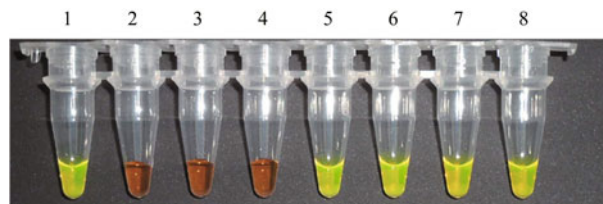

Fig.6 Visual inspection of the *EsrB*-LAMP assay results

All products in the reaction tubes were dyed by adding 0.5 μL of fluorescent dye GeneFinder™ to 25 μL reaction mixture. Tube 1: *E. tarda* 1101 DNA (5 ng); tube 2: water; tubes 3 and 4: DNA extracted from healthy *S. maximus* (No. 2009122104) and *P. solivaceus* (No. 2009122105) intestine tissues, respectively; tubes 5 and 6: DNA extracted from *E. tarda*-infected *S. maximus* (No. 09122103-2 and -4) intestine tissues, respectively; tubes 7 and 8: DNA extracted from *E. tarda*-infected *P. olivaceus* (No. 2009102801-2 and -4) intestine tissues, respectively.

3.5 Practical application of *EsrB*-LAMP to fish samples with fluorescent dye

The LAMP reaction was made visible by direct addition of diluted fluorescent dye to the reaction mixture. Following addition of the dye, the mixture in reaction tubes containing the positive control and DNA extracts from intestine tissues of *E. tarda*-infected *S. maximus* and *P. olivaceus* became green, whereas the mixtures in tubes containing negative control and DNA extracts from healthy fish tissues remained the original orange color (Fig.6). These observations agreed with the results of gel electrophoresis (data not shown).

4 DISCUSSION

Using two additional loop primers in the LAMP method could reduce the reaction time by about half when compared with LAMP without these loop primers (Nagamine et al., 2002; Parida et al., 2004; Tomoko et al., 2004; Yano et al., 2007). However, due to the different characteristics of sequence fragments, it is not always possible to design additional loop primers for the LAMP primer set. To date, no studies of the application of loop primers to LAMP detection of *E. tarda* have been conducted. In this study, we successfully designed a LAMP primer set as well as a pair of loop primers for the *EsrB* gene, which plays an important role in the pathogenicity of *E. tarda*. In addition, we developed an accelerated *EsrB*-LAMP assay for identification of *E. tarda*. The results of this study demonstrate that the *EsrB*-LAMP assay can effectively shorten detection times. Specifically, the amplification product of the *E. tarda* DNA template could be detected after 15 min using 2.5 ng template

DNA. When compared with the hemolysin-LAMP assay developed by Savan et al. (2004), the *EsrB*-LAMP is more sensitive and requires 20 min less time.

Since the Mg^{2+} concentration could affect primer annealing and DNA polymerase activity (Yeh et al., 2005; En et al., 2008; Zhang et al., 2009), we optimized the Mg^{2+} concentration in the LAMP reaction. The results revealed that different Mg^{2+} concentrations strongly influenced the amplification reaction, and that the optimal total Mg^{2+} concentration for *EsrB*-LAMP was 8 mmol/L. These findings were consistent with previously reported ranges of 6–16 mmol/L (Noritaka et al., 2003; Kono et al., 2004; Yeh et al., 2005; Zhang et al., 2009). Temperature optimization tests showed that Bst DNA polymerase could function at 55–69°C, which is also consistent with previous reports (Notomi et al., 2000; Iwamoto et al., 2003; Ihira et al., 2004; Poon et al., 2004).

When compared with conventional PCR, the *EsrB*-LAMP assay showed obviously higher sensitivity for the detection of *E. tarda*. The *EsrB*-LAMP method was 100 times more sensitive than PCR conducted for 35 cycles, which is comparable to the results of previous studies (Tomoko et al., 2004; Mao et al., 2008; Zhang et al., 2009). Analysis of template DNA obtained from nine *E. tarda* strains and thirteen other bacterial strains using the *EsrB*-LAMP assay demonstrated that this method had very high specificity for *E. tarda* strains. Sequence analysis of the LAMP amplification products confirmed the specificity of the primers used for *E. tarda*. Overall, these results indicate that the *EsrB*-LAMP assay is highly reliable for identification of *E. tarda*.

Although the LAMP reaction yielded a white precipitate of magnesium pyrophosphate as a byproduct, as described previously (Mori et al., 2001; Pillai and Bonami, 2006). However, the precipitate might not be clearly visible, which may result in confusion about whether the target DNA was amplified. To address this issue, visual inspection of LAMP amplification products obtained using DNA extracted from the tissue samples of *S. maximus* and *P. olivaceus* as templates was carried out by adding fluorescent dye to the reaction mixture. The positive and negative reactions could be clearly determined with the naked eye based on the color of the reaction mixture. Furthermore, by sealing a drop of fluorescent dye under the lid in advance, we successfully established a patented method (patent number: ZL 2010 1 0147946.2) for staining without opening the tube lid. This method is a good approach to avoid contamination of LAMP products and prevent false positive results, which are a common problem during LAMP tests (Aryan et al., 2010). Thus, the *EsrB*-LAMP assay could also be developed into a closed reaction system for diagnosis, which would be practical for use in the field.

In conclusion, we developed a quick and sensitive *EsrB*-LAMP method for detection of *E. tarda* in culture isolates and fish tissue. This *EsrB*-LAMP assay is the first LAMP protocol to use additional loop primers for the detection of pathogenic *E. tarda*. The LAMP reaction can be conducted under isothermal conditions and does not require any sophisticated instrumentation. Thus, the established *EsrB*-LAMP assays will have a wide range of applications for the detection of *E. tarda* in laboratories, farms, and marine cultures.

5 ACKNOWLEDGEMENT

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