J. Microbiol. Biotechnol. (2015), 25(3), 321–327
http://dx.doi.org/10.4014/jmb.1406.06052

jmb

Development of a Loop-Mediated Isothermal Amplification Assay for Rapid Detection of *Nocardia salmonicida*, the Causative Agent of Nocardiosis in Fish

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Received: June 17, 2014
Revised: September 24, 2014
Accepted: September 28, 2014

First published online
September 29, 2014

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pISSN 1017-7825, eISSN 1738-8872

Nocardia salmonicida is one of the main pathogens of fish nocardiosis. The purpose of this study was to build a loop-mediated isothermal amplification (LAMP) method for the rapid and sensitive detection of *N. salmonicida*. A set of four primers were designed from the 16S–23S rRNA intergenic spacer region of *N. salmonicida*, and conditions for LAMP were optimized as incubating all the reagents for 60 min at 64°C. LAMP products were judged with agar gel electrophoresis as well as with the naked eye after the addition of SYBR Green I. Results showed the sensitivity of the LAMP assay was 1.68×10^3 CFU/ml (16.8 CFU per reaction) and 10-fold higher than that of PCR. The LAMP method was also effectively applied to detect *N. salmonicida* in diseased fish samples, and it may potentially facilitate the surveillance and early diagnosis of fish nocardiosis.

Keywords: Loop-mediated isothermal amplification (LAMP), *Nocardia salmonicida*, rapid detection

Introduction

Nocardiosis is a global fish disease, and has great influence on aquaculture and aquatic products safety [4, 11, 21, 22]. Typical disease signs include nodules in the kidney, spleen, heart, gills, and liver, with or without multiple skin ulcers/nodules. The nocardiosis in fish is mainly caused by *Nocardia seriolae*, *N. salmonicida*, and *N. asteroides* [11, 27]. *Nocardia salmonicida* was first isolated from blueback salmon (*Oncorhynchus nerka*) in 1949 and assigned to the genus *Streptomyces* as *Streptomyces salmonicida* [15]. Later, the bacterium was transferred to the genus *Nocardia* as *Nocardia salmonicida*, based on molecular systematic studies [6]. *N. salmonicida* exists widely in soil and water [24], and it is a conditional pathogen, infecting fish with low immunity. Previous challenge experiment showed *N. salmonicida* can infect zebrafish (*Danio rerio*) and tilapia (*Oreochromis niloticus*) [23].

Over the last few years, nocardiosis has become an emerging fish disease that has caused increasing damage in both freshwater and marine aquaculture systems in Asia [3, 11, 21, 22].

The disease has been reported in more than 20 fish species, with the greatest impact on snakehead (*Ophiocephalus argus*), largemouth bass (*Micropterus salmoides*), golden pomfret (*Trachinotus ovatus*), and large yellow croaker (*Larimichthys crocea*) in China in recent years [5, 20, 21, 27]. Moreover, the nocardial infections are very likely underestimated in Asia because the isolation and culture of nocardial species are quite difficult [11]. Nocardiosis does not show obvious symptoms in the early infection, no effective vaccine has been developed, and there are few effective treatments against this disease because of the antimicrobial resistance [7, 8, 27]. Thus, it is urgent to establish a rapid and sensitive technique to detect the pathogen carrier at early stages and control the disease at the source. Up to now, the rapid detection technologies of

nocardiosis focus mainly on *N. seriolae* [7, 10, 19]. For the rapid detection of *N. salmonicida*, the method of PCR has been established recently [23]. However, this assay requires post-PCR electrophoresis to visually detect the amplified products, and this can be labor-intensive and time-consuming.

In this study, a loop-mediated isothermal amplification (LAMP), method for the rapid and sensitive detection of *N. salmonicida* was established and applied to detect *N. salmonicida* in fish samples. The LAMP assay provided more sensitive and rapid detection of the bacterium than did the PCR assay and conventional culture test, and it may potentially facilitate the surveillance and early diagnosis of fish nocardiosis in the field. This study represents the first report of *N. salmonicida* detection using the LAMP technique.

Materials and Methods

Bacterial Strains and DNA Extraction

A total of eight *N. salmonicida* strains, seven *Nocardia* species, and nine non-*Nocardia* species were used in this study. Some of the bacterial strains were acquired from various diseased fish samples collected from the coast of the South China Sea, and the others were purchased from China General Microbiological Culture Collection Center (CGMCC) (Table 1). After being confirmed by 16S rRNA sequencing, all nocardial species were cultured on an optimized medium (glucose 20 g/l, yeast extract 15 g/l, K_2HPO_4 0.75 g/l, $CaCl_2$ 0.2 g/l (sterilized separately), NaCl 5 g/l, pH 6.5 ± 0.2), and incubated for 72 h at 28°C. Other bacterial cultures were grown on tryptic soy agar (TSA), brain heart infusion broth (BHI), or LB agar (Huankai Co. Ltd., Guangzhou, China) according to the bacterial characteristics [2, 18].

DNA was extracted from cultivated strains using the TIANamp Bacteria DNA Kit (Tiangen Co. Ltd., Beijing, China) according to the manufacturer's instructions. Briefly, gram-positive bacterial cells were treated with lysozyme solution (20 mM Tris (pH 8.0), 2 mM Na_2-EDTA, 1.2% Triton, 20 mg/ml lysozyme) at 37°C over 30 min before DNA extraction. Then, they were subjected to lysis at 70°C for 10 min using GA buffer (200 µl), 20 µl of proteinase K and GB buffer (220 µl), supplied with the kit. After 10 min, 220 µl

Table 1. Bacterial strains used in this study and their sources.

Species	Strain no.	Sources	LAMP-positive strains/number of strains tested
Nocardia spp.			
Nocardia salmonicida	27463	ATCC	1/1
Nocardia salmonicida	4.5220	CGMCC	1/1
Nocardia salmonicida	NS1301, NS1302, NS1303, NS1304, NS1305, NS1306	Diseased Tilapia, Zhanjiang, China	6/6
Nocardia seriolae	ZJ0503	Diseased *Trachinotus ovatus*, Zhanjiang, China	0/1
Nocardia asteroides	19247	ATCC	0/1
Nocardia fluminea	4.5153	CGMCC	0/1
Nocardia fusca	4.1160	CGMCC	0/1
Nocardia flavorosea	4.1175	CGMCC	0/1
Nocardia purpurea	4.1182	CGMCC	0/1
Nocardia violaceofusca	4.1188	CGMCC	0/1
Other isolates			
Streptococcus agalactiae	ZQ0810, ZQ0819, ZQ0910	GDOU	0/3
Vibrio alginolyticus	HY9901, NS0701	GDOU	0/2
Vibrio harveyi	Huang01, Li01	GDOU	0/2
Vibrio parahaemolyticus	VP032, VP1614	GDOU	0/2
Vibrio fluvialis	VF031	GDOU	0/1
Pseudomonas fluorescens	PF041	GDOU	0/1
Aeromonas hydrophila	AH042	GDOU	0/1
Edwardsiella tarda	ET043	GDOU	0/1
Escherichia coli	BL21,DH5α	GDOU	0/2

ATCC: American Type Culture Collection.
CGMCC: China General Microbiological Culture Collection Center.
GDOU: Guangdong Ocean University, China.

of ethanol (100%) was added and the lysate was further incubated for 15 sec. The mixture solution was put into a spin column and centrifuged at 12,000 rpm for 30 sec. After centrifugation, the DNA pellet was washed once with GD buffer (500 µl) and twice with PW buffer (700 µl). Finally, the DNA pellet was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The concentration and purity of the DNA were determined with NanoDrop 2000 (Thermo Fisher Scientific, USA).

Design of LAMP Primers

A set of highly specific primers, F3, B3, FIP, and BIP, were designed according to the 16S-23S rRNA intergenic spacer (ITS) region of *N. salmonicida* strain DSM 40472 (AF536472.1, AF536473.1) [14]. All the LAMP primers were designed by using LAMP primer designing software Primer Explorer ver. 4 (http://primerexplorer.jp/elamp4.0.0/index.html, Eiken Chemical, Japan) (Table 2). All primers were custom synthesized by Shanghai Sangon Biologic Engineering & Technology and Service Co. Ltd, China.

Optimization of LAMP Reaction

The LAMP was performed in 25 µl of a mixture containing 1 µl of the genomic DNA from *N. salmonicida*, 40 pmol (each) of primers FIP and BIP, 5 pmol (each) of primers F3 and B3, 1.6 mmol/l dNTP, and 8 U of *Bst* DNA polymerase (New England Biolabs, Ipswich, MA, USA) with the corresponding polymerase buffer (20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8). The reaction temperatures were optimized by incubating the mixture at 58°C, 60°C, 62°C, 64°C, and 66°C for a predetermined period (60 min). The reaction time was optimized by incubating the mixture for 30, 40, 50, 60, and 70 min at a determined temperature (64°C). The reaction was terminated by heating at 80°C for 5 min. The LAMP products (3 µl) were detected in 2% agarose with ethidium bromide. For visualized detection, 1 µl (1:10) of SYBR Green I (Molecular Probes Inc, Eugene, OR, USA) was added to the LAMP product and the result was observed with the naked eye.

Specificity of LAMP Assay

To determine the specificity of the LAMP assay, LAMP was carried out at 64°C for 60 min with different DNA templates from the 17 bacteria species in Table 1. Reaction mixture without DNA template was included as a negative control. Each DNA sample of the tested strains was examined in triplicate. The products were separated by 2% agarose gel electrophoresis, and the target bands were visualized by staining with ethidium bromide.

Sensitivity of LAMP Assay

A 10-fold serial dilution of *N. salmonicida* DNA, starting from 2.83 ng/µl to 0.283 pg/µl, was used as the template for LAMP following the predetermined conditions described above. A reaction mixture without DNA template was included as a negative control. The products were electrophoresed on 2% agarose gel. To compare the detection sensitivity of LAMP and PCR, a serial dilution of *N. salmonicida* DNA was subjected to PCR using primers PCR-F (5′-CAGTCCGATATCGCGGTGAA-3′) and PCR-R (5′-GACCTACTCGACCAAGTGGC-3′) [20]. The sensitivity tests of the LAMP and PCR assay were conducted in triplicate, and the detection limits were defined as the last positive dilutions. In parallel, to enumerate the bacteria, 100 µl aliquots of *N. salmonicida* suspensions were spread on the optimized medium (1% agar) and incubated at 28°C for 72 h. Colonies were counted at the dilution yielding 30–300 colony-forming units (CFU), and CFU per milliliters of suspension was calculated.

PCR Assay

PCR was carried out according to the method of Xia *et al.* [23]. Briefly, PCR was carried out in a total reaction volume of 25 µl with 1 µl of the genomic DNA, 2.5 µl of the buffer solution (100 mmol/l Tris-HCl, 500 mmol/l KCl, 15 mmol/l $MgCl_2$, pH 8.3), 2 µl (10 pmol/l) of a pair of PCR-F and PCR-R primers, 2 µl of dNTP mixture (2.5 mmol of each dNTP), and 1.25 U Taq DNA polymerase (Takara, Shiga, Japan). The PCR conditions consisted of an initial denaturation of 94°C for 5 min and 30 cycles of 94°C for 30 sec, 59°C for 30 sec, 72°C for 30 sec, and a final extension of 72°C for 5 min in a S1000 thermal cycler (Bio-Rad, Hercules, CA, USA). The amplified products (3 µl) were then analyzed by 1% agarose gel, and the target bands were visualized by staining with ethidium bromide.

Application of LAMP to Detect *N. salmonicida* Infection in Fish Tissues

To compare the difference among the LAMP, PCR, and culture

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

Purpose	Primer	Type	Sequence (5′-3′)	Length (nt)
LAMP	FIP	Forward inner (F1c-TTTT-F2)	TTCCACCCATGAGCTCCAGCTTTTTTGGTGAGTGGTTGGCAGA	43 (F1c:20nt, F2:19nt)
	BIP	Backward inner (B1c-TTTT-B2)	CTTCGCATCATGGTGTCGGCTTTTTCGCGTGTTCTTTCAGGACC	44 (B1c:21nt, B2:19nt)
	F3	Forward outer	GCGCATTCCCAACACAGG	18
	B3	Backward outer	CGGGAGGATCGTTATCTTGC	20
PCR	PCR-F	Forward	CAGTCCGATATCGCGGTGAA	20
	PCR-R	Reverse	GACCTACTCGACCAAGTGGC	20

tests on *N. salmonicida* detection in infected fish, a group of adult zebrafish ($n = 50$, weighed 0.25 to 0.35 g) and tilapia ($n = 6$, average weight about 100 g) were injected intraperitoneally with 10 µl and 100 µl of *N. salmonicida* suspension (8.4×10^8 CFU/ml), respectively. Control zebrafish ($n = 10$) and tilapia ($n = 5$) were inoculated with 10 µl and 100 µl PBS, respectively. After 72 h post infection, a total of five zebrafish and three tilapia with obvious clinical signs were chosen randomly. Kidney tissues were aseptically excised from both infected and control fish, and then plated on Lowenstein-Jensen medium and incubated at 28°C for culture test. At the same time, the kidney samples were used for DNA extraction with the TIANamp Genomic DNA Kit (Tiangen Co. Ltd., Beijing, China). DNA obtained from fish tissues were analyzed by LAMP and PCR following the conditions described earlier. The products obtained were subjected to 2% agarose gel electrophoresis.

Results

Optimized LAMP Reaction

The LAMP products were formed at 58°C, 60°C, 62°C, 64°C, and 66°C, and 64°C was chosen as the optimal temperature because of the clarity of the bands (Fig. 1A). With regard to the reaction time, LAMP products were observed at 30, 40, 50, 60, and 70 min at 64°C (Fig. 1B). To ensure positive detection of the lower concentration of template in the system, we chose 60 min as the optimal time. On the basis of the above analyses, the conditions of amplification were optimized as 60 min at 64°C.

Specificity of the LAMP Assay

The specificity of the LAMP method was tested using DNA templates extracted from other seven nocardial species and nine non-nocardial species (Table 1). After incubation at 64°C for 60 min, all eight strains of *N. salmonicida* were positively detected, whereas the other bacterial strains did not produce any amplification by LAMP (Fig. 2). These results indicate the LAMP assay is highly specific for *N. salmonicida* in this study. The LAMP products in positive tubes appeared green after the addition of SYBR Green I dye, whereas the original orange color of SYBR Green I did

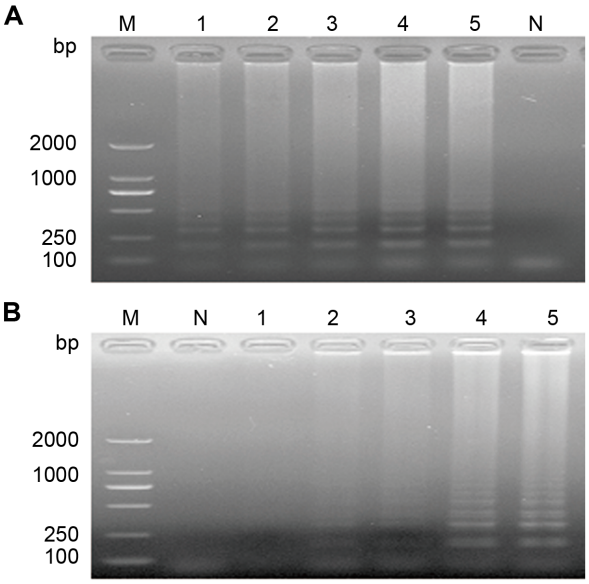

Fig. 1. Determination of the optimal temperature and time of LAMP.
(**A**) Determination of the optimal temperature. Lane M, DL2000 DNA marker; lanes 1–5, LAMP carried out at 58°C, 60°C, 62°C, 64°C and 66°C, respectively; lane N, negative control. (**B**) Determination of the optimal time. Lane M, DL2000 DNA marker; lane N, negative control; lanes 1–5, LAMP carried out for 30, 40, 50, 60 and 70 min, respectively.

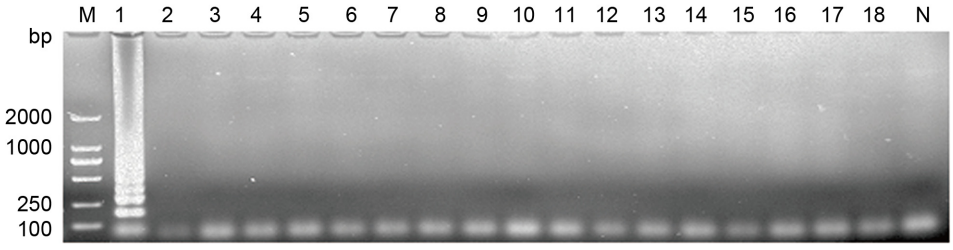

Fig. 2. Specificity of the LAMP assay for *Nocardia salmonicida*.
Lane M, DL2000 DNA marker; lane 1, *N. salmonicida* strain ATCC 27463; lane 2, *N. seriolae* strain ZJ0503; lane 3, *N. asteroides* strain CGMCC 4.1165; lane 4, *N. fluminea* strain CGMCC 4.5153; lane 5, *N. fusca* strain CGMCC 4.1160; lane 6, *N. flavorosea* strain CGMCC 4.1175; lane 7, *N. purpurea* strain CGMCC 4.1182; lane 8, *N. violaceofusca* strain CGMCC 4.1188; lane 9, *Streptococcus agalactiae* strain ZQ0810; lane 10, *Edwardsiella tarda* strain ET043; lane 11, *Aeromonas hydrophila* strain AH042; lane 12, *Pseudomonas fluorescens* strain PF041; lane 13, *Vibrio harveyi* strain Huang01; lane 14, *Vibrio parahaemolyticus* strain VP032; lane 15, *Vibrio alginolyticus* strain HY9901; lane 16, *Vibrio fluvialis* strain VF031; lane 17, *Escherichia coli* strain BL21; lane 18, *Escherichia coli* strain DH5α; lane N, negative control.

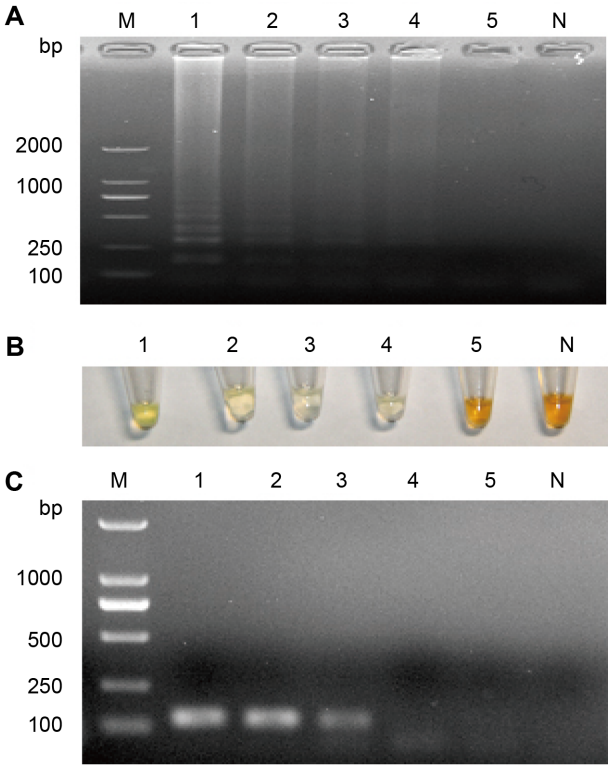

Fig. 3. Sensitivity test for LAMP and PCR using the same 10-fold series dilutions of *N. salmonicida* DNA (10^{-1} to 10^{-5} dilution equivalent to 16,800~1.68 CFU per reaction).
(**A**) Result of agarose gel electrophoresis of LAMP products. Lane M, DL2000 DNA marker; lanes 1–5, reaction carried out using 10^{-1} to 10^{-5} dilutions of *N. salmonicida* DNA (2.83 ng/μl to 0.283 pg/μl), respectively; lane N, negative control. (**B**) Detection of LAMP products, after addition of SYBR Green I, with the naked eye. Tubes 1–5, the LAMP-amplified products carried out using 10^{-1} to 10^{-5} dilutions of *N. salmonicida* DNA (2.83 ng/μl to 0.283 pg/μl), respectively; tube N, negative control. (**C**) Result of agarose gel electrophoresis of PCR products. Lane M, DL2000 marker; lanes 1–5, reaction was carried out using 10^{-1} to 10^{-5} dilutions of *N. salmonicida* DNA (2.83 ng/μl to 0.283 pg/μl), respectively; lane N, negative control.

not change in the negative control tubes (Fig. 3B). Color change was observable with the naked eye.

Sensitivity of the LAMP Assay

To evaluate the sensitivity of the LAMP assay for the detection of *N. salmonicida*, a 10-fold serial dilution of the *N. salmonicida* DNA was used for the LAMP and PCR assays. The LAMP reaction was able to detect *N. salmonicida* up to 1.68×10^3 CFU/ml (16.8 CFU per reaction, Fig. 3A). However, PCR could only detect *N. salmonicida* up to 1.68×10^4 CFU/ml (168 CFU per reaction, Fig. 3C). The

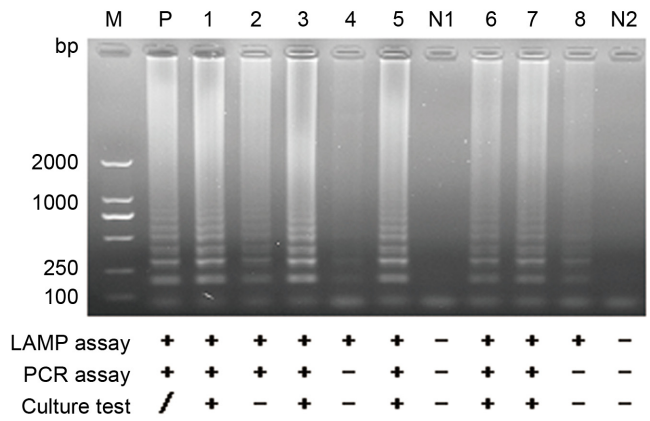

Fig. 4. Detection of *N. salmonicida* by LAMP assay, PCR assay, or culture test, from infected and control fish samples.
Lane M, DL2000 DNA marker; Lanes P, positive control (DNA extracted from *N. salmonicida* suspensions); lanes 1–5, infected zebrafish No. 1–5; Lanes N1, control zebrafish; lanes 6–8, infected tilapia No. 1–3; Lanes N2, control tilapia; "+", represents a positive result; "–", represents a negative result; "/", no test performed.

results indicate the sensitivity of LAMP was 10-fold higher than PCR.

Application of LAMP to Detect *N. salmonicida* Infection in Fish Tissues

To examine whether the LAMP assay could be applied to detect the *N. salmonicida* infection in fish tissues, the LAMP was carried out in infected or control fish samples. As shown in Fig. 4, LAMP could detect *N. salmonicida* in all infected fish with obvious clinical signs (lanes 1–8), where the total detection rates for the LAMP assay were 100%. The control sample did not show any presence of LAMP bands (lanes N1 and N2). From the eight fish samples, six and five samples were detected as positive by the PCR assay and culture test, respectively. Total detection rates for the PCR assay and culture test were 75% (6/8) vs. 62.5% (5/8). In comparison with conventional PCR methods and culture test, LAMP yielded a better detection rate.

Discussion

Nocardiosis is a systemic granulomatous disease, and its damage to the fish industry has been increasing in recent years [11]. The most effective prevention of this disease is the detection and elimination of carrier fish from fish populations [7]. Early, accurate, and rapid detection methods are critical for the successful management of this disease in aquaculture. The isolation and culture of nocardial pathogen

were difficult, and Labrie *et al.* [11] reported that *Noardia* sp. could be recovered by the culture method in only 55% of nocardiosis cases in their investigation in South East Asia. As shown in Fig. 4, *N. salmonicida* was isolated by the culture test from only five of the eight fish samples that were all positive with the LAMP assay. Thus, the bacterial culture test cannot be a reliable detection method to diagnose fish nocardiosis in consideration of the low detection rate.

LAMP is a sensitive method, which can amplify a few copies of DNA to a magnitude of 10^9 copies in less than 1 h [12, 13]. The LAMP reaction is done under isothermal conditions and does not require expensive equipment. Moreover, the results of the LAMP assay can be interpreted using a simplified optical system or even with the naked eye. By taking advantage of this technology, the LAMP assay has been successfully applied to the diagnosis of some fish diseases [1, 2, 16, 17, 26]. The intergenic spacer region between the 16S–23S rRNA genes is an excellent candidate for the identification of bacterial species. In this study, a set of highly specific primers was designed according to the 16S–23S rRNA intergenic spacer region of *N. salmonicida*, and a novel LAMP assay was developed as a rapid and simple detection tool for the specific diagnosis of *N. salmonicida*. The LAMP assay requires only a simple water bath or heating block to incubate the reaction mixture at 64°C for 60 min before the reaction products are visualized.

In addition to its higher specificity, the LAMP assay for *N. salmonicida* identification showed sufficient sensitivity, as it could detect *N. salmonicida* up to 1.68×10^3 CFU/ml, whereas with PCR the detection of *N. salmonicida* was possible up to 1.68×10^4 CFU/ml. This result indicates that LAMP was 10-fold more sensitive than the PCR assay, which is in complete accordance with the results reported by Itano *et al.* [7] for the detection of *N. seriolae*. The greater sensitivity could be because of its high amplification efficiency and no time loss for thermal change under isothermal conditions. As shown in Fig. 4, false-negative happened in two *N. salmonicida*-infected samples by PCR assay. It has been reported that the false-negative in the PCR assay can occur by inhibition of DNA amplification due to the components in the tissue samples, and PCR assay is known to be more sensitive than the LAMP assay for this inhibitory effect [9, 12, 25]. In addition, gram-positive bacteria like *N. salmonicida,* which can form compact and solid bacterial colonies, are difficult to get high DNA concentration from during DNA extraction. The high sensitivity and low false-negative rate make LAMP a better choice than PCR for the diagnosis of *N. salmonicida* in cases when lower concentrations of bacteria or DNA are expected.

In conclusion, the LAMP assay described in this study represents a new sensitive, specific, and rapid protocol for the detection of *N. salmonicida*. This method provides an important diagnostic tool for the detection of *N. salmonicida* infection in the laboratory. For further use in the field, more data are needed by applying the established LAMP assay to the field samples from natural outbreaks.

Acknowledgments

This study was supported by the International S&T Cooperation Projects of Guangdong Province (2012B050600029), the Project of Internation as well as Hongkong, Macao & Taiwan Science and Technology Cooperation Innovation Platform in Universities in Guangdong Province (2013gjhz0008) and the Projects for the Innovation and Development in Higher Education of Guangdong Province (2014KTSCX075).

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