Development of a sensitive and specific LAMP PCR assay for detection of fish pathogen *Lactococcus garvieae*

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ABSTRACT: Based on use of a loop-mediated isothermal amplification (LAMP) identification protocol, this study attempted to detect *Lactococcus garvieae* in fish by using primer sets designed from an *L. garvieae* alpha/beta fold family hydrolase gene. Reaction time and temperatures were optimized for 60 min at 60°C with the resulting amplicons visualized by adding SYBR Green I to the reaction tube. The assay specificity was assessed using 45 different bacterial strains. Positive results were observed in all 30 *L. garvieae* isolates from various aquatic animals. No false-positive results were observed in 15 non-*L. garvieae* strains. The detection limit of the LAMP assay was 10-fold more sensitive than the conventional polymerase chain reaction (PCR) targeting 16S rDNA when using purified *L. garvieae* DNA. The detection limit of the LAMP assay was approximately 300 colony-forming units (CFU) using crude bacterial lysates, 100-fold more sensitive than PCR. Furthermore, *L. garvieae* in spleen, kidney and brain of experimentally challenged tilapia and grey mullet were detected using this optimized LAMP assay. Results of this study demonstrate the effectiveness of LAMP in providing a rapid yet simple test for detecting *L. garvieae* in fish.

KEY WORDS: Lactococcosis · Alpha/beta fold family · Hydrolase gene · Loop-mediated isothermal amplification · Polymerase chain reaction · SYBR Green I

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INTRODUCTION

Lactococcus garvieae has been isolated as causative agent of disease in rainbow trout *Oncorhynchus mykiss* (Chang et al. 2002), yellowtail *Seriola quinqueradiata*, tilapia *Oreochromis* sp. (Kusuda et al. 1991), amberjack *S. dumerili*, kingfish *S. lalandi* (Kawanishi et al. 2005), wild wrasse *Coris aygula* (Colorni et al. 2003), black rockfish *Sebastes schlegeli* (Kang et al. 2004), grey mullet *Mugil cephalus* (Chen et al. 2002) and in giant freshwater prawn *Macrobrachium rosenbergii* (Chen et al. 2001). In addition to aquatic animals, *L. garvieae* has been isolated from other homoiothermic and poikilothermic animals such as cows, buffalos, cats and dogs

(Pot et al. 1996, Carvalho et al. 1997, Devriese et al. 1999). *L. garvieae* is also a zoonotic pathogen, occurring rarely and with a low virulence in human infections (Wang et al. 2007). This disease infects farmed fish during the summer when the water temperature exceeds 20°C (Park et al. 1997). *L. garvieae* induces a fatal hemorrhagic septicemia of fish. *L. garvieae* is reported to be the major pathogen in bacterial infections of cultured grey mullet, occurring mainly from May to September in Taiwan. Epizootics with up to 10% mortality were recorded on some Taiwanese grey mullet farms (Chen et al. 2002, Lee et al. 2006). Additionally, the gross pathology of lactococcosis initially appears with rapid and general anorexia, melanosis, lethargy, loss of orientation and erratic

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swimming. However, similar clinical signs are also found in *Streptococcus* sp. infections (Eldar & Ghitino 1999).

Molecular detection methods of *Lactococcus garvieae* have been developed, including polymerase chain reaction (PCR) (Zlotkin et al. 1998, Aoki et al. 2000, Pu et al. 2002) and 16S rRNA and *sodA* sequence analysis (Fihman et al. 2006). However, although considered rapid and specific methods for detection, these require expensive equipment and other costly materials (Gunimaladevi et al. 2005).

A method of amplifying nucleic acid sequences with high specificity, sensitivity and rapidity under isothermal conditions (60 to 65°C), loop-mediated isothermal amplification (LAMP) has been developed more recently (Notomi et al. 2000) and applied to fish pathogen detection (Caipang et al. 2004, Savan et al. 2004, 2005, Yeh et al. 2005, Itano et al. 2006). The assay uses a set of 4 or 6 primers that create a target-specific stem loop DNA structure during initial amplification steps; subsequent LAMP auto-cycling is performed by the *Bst* DNA polymerase large fragment (Notomi et al. 2000). Since it does not require complicated equipment, the LAMP method provides a cost-effective, simple, and rapid (producing a result within 60 min) DNA amplification method.

Recently, a fosmid library of *Lactococcus garvieae* from grey mullet has been constructed and many new genes of *L. garvieae* were cloned (M. A. Tsai et al. unpubl. data). The clone flg08-6 contained an alpha/beta fold family hydrolase gene that is also present in other bacteria. This gene showed a relatively high level of nucleotide differences between *L. garvieae* and closely related *Lactococcus* species. Thus, we were interested in developing a rapid, simple and sensitive LAMP method based on the alpha/beta fold family hydrolase gene for detecting *L. garvieae* in fish tissues. A set of 4 distinct primers in the LAMP assay were tested for specificity and sensitivity. Additionally, the sensitivity of the LAMP method was compared with that of the conventional PCR method. Finally, the LAMP assay was used to detect *L. garvieae* from experimentally challenged tilapia and grey mullet.

MATERIALS AND METHODS

Bacterial strains

A total of 45 *Lactococcus garvieae* strains and other bacterial species from Bioresource Collection and Research Center (food industry research and devel-

opment institute) were used in this study (Table 1). These included *L. garvieae* reference strains, and 18 strains isolated from grey mullet, basjet mullet, tilapia, grouper, rainbow trout, striped bass, giant freshwater prawn and bullfrog in Taiwan, an *L. garvieae* strain isolated from a patient with gastrointestinal disease in Taiwan and additional *L. garvieae* strains isolated from yellowtail and tuna in Japan and Spain. All strains were confirmed as *L. garvieae* based on PCR with 2 species-specific primer sets, pLG-1 and pLG-2, and SA1B10-1-F and SA1B10-1-R, as described by Zlotkin et al. (1998) and Aoki et al. (2000), respectively. Non-*L. garvieae* strains (Table 1) included *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *L. raffinolactis* and pathogenic bacteria of fish including *Streptococcus iniae*, *S. agalactiae*, *S. dysgalactiae*, *S. uberis*, *Nocardia seriolae*, *N. salmonicida*, *Edwardsiella tarda*, *Aeromonas hydrophila* subsp. *hydrophila*, *Vibrio vulnificus*, and *V. parahaemolyticus*. These bacterial species are important pathogens in tilapia, grey mullet and other fish. *L. garvieae* strain ATCC 43921 from the American Type Culture Collection was used as a positive control for PCR and LAMP assays. *L. garvieae* was cultured on Todd-Hewitt Agar (THA; Difco), and Todd-Hewitt Broth (THB; Difco) at 25°C with moderate shaking at 125 rpm. Other bacteria were grown on Brain Heart Infusion Broth (BHI; Difco) medium with 1.5% (w/v) agar at 25°C.

Preparation of genomic DNA

DNA was extracted from *Lactococcus garvieae* and other bacteria using the method of Chen et al. (2001). DNA from overnight cultures (10 ml) was isolated by phenol/chloroform extraction, followed by precipitation with isopropanol. The DNA pellet was then dissolved in 100 µl sterilized distilled water and stored at –20°C until use. Quantification of genomic DNA was performed using the optical density of DNA (260/280 nm) measured by a UV-Vis spectrophotometer. The DNA samples were diluted to 50 ng µl⁻¹.

Preparation of crude DNA lysate

Crude DNA lysate was prepared according to the boiled-extraction method of Aoki et al. (2000), as follows: bacteria were grown in THB incubated at 25°C for 24 h and washed 3 times with phosphate-buffered saline (PBS, pH 7.2). After centrifugation

Table 1. *Lactococcus garvieae* and other bacterial strains used in this study. Results (positive or negative) of amplification by alpha/beta fold family hydrolase gene loop-mediated isothermal amplification (LAMP) assay are shown. ATCC: American Type Culture Collection; BCRC: Bioresource Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan

Strain	No. of bacterial isolates	Source or reference	Country	GenBank accession nos. (strain nos.)	LAMP
<i>L. garvieae</i> strains (n = 30)					
<i>L. garvieae</i>	10	Grey mullet	Taiwan	JF766366 (FLG15); JQ087264 (FLG19); JQ087265 (FLG18); JQ087266 (FLG20); JQ087267 (FLG58)	+
<i>L. garvieae</i>	1	Basket mullet	Taiwan	JQ087268 (FLG31)	+
<i>L. garvieae</i>	2	Tilapia	Taiwan		+
<i>L. garvieae</i>	1	Grouper	Taiwan		+
<i>L. garvieae</i>	1	Striped bass	Taiwan	JQ087269 (FLG98)	+
<i>L. garvieae</i>	1	Bullfrog	Taiwan		+
<i>L. garvieae</i>	3	Giant freshwater prawn	Taiwan		+
<i>L. garvieae</i>	1	Rainbow trout	Taiwan		+
<i>L. garvieae</i>	1	Human (Wang et al. 2007)	Taiwan		+
<i>L. garvieae</i>	3	Yellowtail	Japan	JQ087270 (C1); JQ087271 (C2); JQ087272 (C3)	+
<i>L. garvieae</i>	3	Yellowtail	Japan	MT2431; MT2433; MT2434	+
<i>L. garvieae</i>	1	Tuna	Japan	JQ087273 (KGLA0412)	+
<i>L. garvieae</i>	2	Rainbow trout	Spain	350; 356	+
<i>L. garvieae</i>		ATCC 43921 (Cow)	UK	JQ087274	+
Non-<i>L. garvieae</i> strains (n = 15)					
<i>L. lactis</i> subsp. <i>lactis</i>		BCRC 10791			–
<i>L. lactis</i> subsp. <i>cremoris</i>		BCRC 11067			–
<i>L. raffinolactis</i>		BCRC 14039			–
<i>Streptococcus iniae</i>		BCRC 14744			–
<i>S. agalactiae</i>		BCRC 10787			–
<i>S. dysgalactiae</i>		BCRC 12577			–
<i>S. uberis</i>		BCRC 12579			–
<i>S. pyogenes</i>		BCRC 10797			–
<i>Staphylococcus aureus</i> subsp. <i>aureus</i>		BCRC 10781			–
<i>Nocardia seriolae</i>		BCRC 13745			–
<i>N. salmonicida</i>		BCRC 12441			–
<i>Edwardsiella tarda</i>		BCRC 10670			–
<i>Aeromonas hydrophila</i> subsp. <i>hydrophila</i>		BCRC 13018			–
<i>Vibrio vulnificus</i>		BCRC 13846			–
<i>V. parahaemolyticus</i>		BCRC 12864			–

(3500 × *g* for 10 min), the pellet was adjusted to an optical density of 1.0 at 610 nm with PBS and colony-forming units (CFU) counted following plating of bacterial suspension on THA. In the above method, the bacterial suspension was again centrifuged briefly in a microcentrifuge tube at the maximum speed. The pellet was then resuspended in distilled water, boiled at 100°C for 10 min and centrifuged again briefly at the maximum speed. The supernatant containing the template DNA was used for LAMP and PCR.

Detection of *Lactococcus garvieae* by 16S rDNA PCR

Lactococcus garvieae detection using a PCR targeting 16S rRNA (Zlotkin et al. 1998) was performed to confirm identity of bacterial isolates and also to compare its detection limit with that of the LAMP assay. 16S rDNA PCR was performed on a thermal cycler (Biorad) with reaction mixtures containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 200 μM deoxynucleotide triphos-

phate, 10 µM primers pLG-1 and pLG-2, 2 µl (50 ng) of template DNA, and 0.75 U of Blend *Taq*[®] DNA polymerase (Toyobo) in a final volume of 25 µl. Amplification conditions consisted of an initial denaturation step at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1.5 min and a final extension step of 72°C for 10 min. The PCR products were resolved by electrophoresis on a 2% agarose gel.

Alpha/beta fold family hydrolase gene analysis

The sequence of *Lactococcus garvieae* alpha/beta fold family hydrolase gene obtained from clone Flg08-6 of a fosmid library was utilized to design forward and reverse PCR primers Lgf08-6-823F (5'-TTT GTT GCT GCA GGT TAT CG-3') and Lgf08-6-823R (5'-AAT TGG TGG CGC TTA TTT TG-3'), using Primer 3 (frodo.wi.mit.edu/primer3/). The alpha/beta fold family hydrolase gene was amplified from 11 *L. garvieae* strains, from a total of 10 different fish species from Taiwan and Japan and 1 reference strain, ATCC 43921, from cow. The PCR was performed on a Thermal Cycler (BioRad) with reaction mixtures containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 200 µM deoxynucleotide triphosphate, 10 µM each primer, 2 µl (50 ng) of template DNA, and 0.625 U of Blend *Taq*[®] DNA polymerase (Toyobo) to a final volume of 50 µl. The following cycling program was run: preheating at 96°C for 5 min and 33 cycles of denaturing at 96°C for 50 s, annealing at 48°C for 50 s, and extension at 72°C for 1 min and followed by a final cycle with a 10 min extension step at 72°C. The expected fragment size is 823 bp, which includes the alpha/beta fold family hydrolase full length gene (591 bp). The PCR products (5 µl) were subjected to electrophoresis (35 min, 100 V) in 2% agarose gel (LE agarose; SeaKem) with 0.5× TBE buffer (Sigma-Aldrich) containing 90 mM Tris, 90 mM borate and 2 mM EDTA (pH 8.3) and visualized using ethidium bromide staining. Amplified PCR products were purified using a gel/PCR DNA fragments extraction kit (Geneaid Biotech). Sequencing was carried out by Tri-I Biotech, Taiwan, using standard procedures. Sequencing reactions were performed using BigDye[®] Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems) with the primers Lgf08-6-823F and Lgf08-6-823R and run on the ABI 3730 DNA analyzer (Applied Biosystems). Raw sequence data were assembled and edited using the SeqMan

module within the DNASTar software package. Multiple sequence alignments were generated using CLUSTALW 2.0 (www.ebi.ac.uk/Tools/clustalw2/).

LAMP primer design

Primers for LAMP were designed to target the alpha/beta fold family hydrolase gene from *Lactococcus garvieae* (accession number JF766366), by using LAMP PrimerExplorer V4 software program (primer-explorer.jp/e/v4_manual/index.html) (Fig. 1). The forward inner primer (FIP: 5'-AGA GCT TTT TAC GGA CGG AAA GTA AAT GTA GGT GCT TGA TCC T-3') consisted of a complementary sequence of F1c and a sense sequence of F2 (Fig. 1), without any linker sequence in between. Similarly, the backward inner primer (BIP: 5'-ATA TGC CAT AAT AGT CGC TGC TCA ATG CCC TGA AAC TTT ATC GA-3') was a direct combination of a complementary sequence of B1c and a sense sequence of B2 (Fig. 1). The 2 outer primers, forward outer primer and backward outer primer, were F3 and B3, respectively (Fig. 1). The LAMP product generated from the alpha/beta fold family hydrolase of *L. garvieae* was 180 bp long.

Determination of conditions for LAMP

LAMP reactions were conducted using a thermal cycler (BioRad) with reaction mixtures containing 1.6 µM each of the inner primer, FIP and BIP, 0.2 µM each of the outer primers (F3 and B3 primer), 1.5 mM dNTPs, 8 U of *Bst* polymerase (New England Biolabs) together with the supplied buffer (Thermopol buffer containing 2 mM of MgSO₄) and 2 µl (50 ng) of the extracted template DNA in a final volume of 25 µl. The mixture was incubated at 65.0, 63.5, 61.7, 59.8, 57.9, 56.0, 54.1 and 52.2°C for 1 h, and the reaction was terminated by heating at 80°C for 2 min. Time-specific amplification for 15, 30, 45, and 60 min at 60°C was undertaken with serial 10-fold dilutions (from 100 fg to 100 ng) of purified DNA. The amplified products were electrophoresed in 2% agarose gel.

Specificity of LAMP

The specificity of LAMP primers was examined using different *Lactococcus garvieae* and non-*L. garvieae* bacterial strains (Table 1). These latter bacteria are important pathogens in tilapia, grey mullet

	F3	nt
JF766366	AAAATCAGATAATTACCAAGTGTGTTTACCTGTTTGCCCATCTAACCAATCGGGCGCTTT	414
FLG18		414
FLG19		414
FLG20		414
FLG31		414
FLG58		414
FLG98		414
C1		414
C2		414
C3		414
KGLA0412		414
ATCC43921	A	414
AP009332 (LG)		414
AP009333 (LG)		414
CP002365 (LLSL)	...TA.T..C.GC..TGTC.....C..A.A..A..TG.G....T.C..A...	420
NC 008527.1 (LLSC)	...GAT...TGTT.....C..ATA...A...T.....G..A.CT.A...	420
	F2	F1c
JF766366	CATAAATGTAGGTGCTTGATCCTCTGTTACGATGGCCGCTAACTTTCCGTCCGTA AAAAG	474
FLG18		474
FLG19		474
FLG20		474
FLG31		474
FLG58		474
FLG98		474
C1		474
C2		474
C3		474
KGLA0412		474
ATCC43921		474
AP009332 (LG)		474
AP009333 (LG)		474
CP002365 (LLSL)	G.AG..A..T..G..C...T...A.TT..T..T.A..CA..T...A...T.A	480
NC 008527.1 (LLSC)	A.A...A..T..C...G..T...G.TT.CT..TCT..CA...T...A...T.A	480
	B1c	B2
JF766366	CTCTTCATATGCCATAATAGTCGCTGCTCCCATCGAGTGTCCAACCAATACTGGTCGATA	534
FLG18		534
FLG19		534
FLG20		534
FLG31		534
FLG58		534
FLG98		534
C1		534
C2		534
C3		534
KGLA0412		534
ATCC43921		534
AP009332 (LG)		534
AP009333 (LG)		534
CP002365 (LLSL)	T.....A.....T..T..C..A.....A..G...TT..G..AACATTT..T	540
NC 008527.1 (LLSC)	T.....A.....T..T..C..A.....T..A.....TT..G..TGACGTTT..T	540
	B3	
JF766366	AAGTTTCAGGGCATTTCATCAACTCTTCCAAATCCGTCGCCAGACGATGTAAGGTCAT---	591
FLG18		591
FLG19		591
FLG20		591
FLG31		591
FLG58	A.....	591
FLG98		591
C1	A.....	591
C2	A.....	591
C3	A.....	591
KGLA0412	A.....	591
ATCC43921	A.....	591
AP009332 (LG)	A.....	591
AP009333 (LG)	A.....	591
CP002365 (LLSL)	T..A..GT..AATAG..CA..T..T...TT..GG...A..T...A...G....A...AGCC	600
NC 008527.1 (LLSC)	T...G..A..TGG..CA....T...TT.....A..G....AT.....A...AGCC	600

Fig. 1. Multiple nucleotide alignment of partial sequences of the alpha/beta fold family hydrolase gene of *Lactococcus garvieae* (GenBank accession no. JF766366), other *L. garvieae* strains and *L. lactis*. *L. garvieae* fish isolates: FLG18 (grey mullet, Taiwan), FLG19 (grey mullet, Taiwan), FLG20 (grey mullet, Taiwan), FLG31 (basket, Taiwan), FLG58 (grey mullet, Taiwan), FLG98 (striped bass, Taiwan), C1 (yellowtail, Japan), C2 (yellowtail, Japan), C3 (yellowtail, Japan), KGLA0412 (tuna, Japan), ATCC 43921 (cow, UK), *L. garvieae* (GenBank accession no. AP009332), *L. garvieae* (GenBank accession no. AP009333) *L. lactis* subsp. *lactis* (GenBank accession no. CP002365) and *L. lactis* subsp. *cremoris* (GenBank accession no. NC 008527.1). Annealing sites of primers F3, B3, F2, F1c, B1c and B2 are underlined. The forward inner primer (FIP) used in the present study consisted of a complementary sequence of F1c and a sense sequence of F2, without any linker sequence in between. Similarly, the backward inner primer (BIP) was a direct combination of a complementary sequence of B1c and a sense sequence of B2

and other fish. *L. garvieae* strains isolated from additional fish species and geographical regions, and from humans (Table 1) (*L. garvieae* strains 350 and 356 were provided by Prof. A. E. Toranzo, Universidad de Santiago de Compostela), were also examined by LAMP. The content of the LAMP reaction was as described in the previous subsection, using 2 µl (100 ng of purified DNA) and 2 µl sterilized distilled water replaced DNA in negative controls. LAMP reactions were carried out at 60°C for 1 h.

Sensitivity comparison of LAMP and PCR

The detection limits of the LAMP and PCR assay for *Lactococcus garvieae* were determined as follows: bacterial crude lysate and purified genomic DNA were serially diluted 10-fold (3 to 3×10^6 CFU and 200 fg to 200 ng) and subjected to testing by 16S rDNA PCR and LAMP under reaction and cycling conditions as described previously for each assay. Each dilution was tested in triplicate. The amplified products generated by both traditional PCR of 16S rDNA and LAMP assay for the alpha/beta fold family hydrolase gene were electrophoresed in 2% agarose gel. Additionally, the amplification products generated by LAMP were detected by visual inspection using SYBR Green I according to the method of Iwamoto et al. (2003). Maximum dilution was considered as minimum sensitivity for LAMP and PCR detection.

Infected fish tissue evaluation

Healthy grey mullet (35 ± 4 g) and tilapia *Oreochromis niloticus* (60 ± 5 g) were obtained from a farm in Chai-Yi and Tainan, Taiwan, respectively, and held at a density of 100 fish in a continuously aerated 500 l aquarium containing 450 l of water at approximately 25°C for 7 d until they were acclimatized. The fish were fed twice daily with commercial fish pellets and waste was removed daily. Fish were anaesthetized using benzocaine (Sigma) prior to inoculation. 45 tilapia ($n = 15$, in triplicate) and 15 grey mullet ($n = 5$, in triplicate) were inoculated by intraperitoneal injection with 10^8 CFU of *Lactococcus garvieae* per fish. Uninfected fish (10 tilapia and 5 grey mullet) were inoculated with PBS (pH 7.2). Fish were lethally anaesthetised with benzocaine 24 h post infection. The skin of fish was surface sterilized by swabbing with 70% ethanol before being cut open to reveal underlying tissue.

Bacteria were reisolated from spleen, kidney and brain on THA, and PCR using pLG-1 and pLG-2 primers used to confirm identification of the bacterial isolates. In addition the above tissues were aseptically excised, and DNA was obtained using the method of Chen et al. (2001). Extracted DNA from uninfected fish was used as negative control. The LAMP and PCR assays followed the predetermined conditions described above and were carried out in triplicate. *L. garvieae* genomic DNA was used as positive control in PCR and LAMP assays. The products obtained were subjected to 2% agarose gel electrophoresis.

RESULTS

Analysis of alpha/beta fold family hydrolase gene

An 823 nucleotide PCR product was obtained from the alpha/beta gene of 11 *Lactococcus garvieae* strains, from a total of 10 different fish species from Taiwan and Japan and 1 reference strain from cow (ATCC 43921), using the primers Lgf08-6-823F and Lgf08-6-823R. The PCR product contains a 591 bp full alpha/beta gene. These sequences were submitted to the GenBank database under accession numbers JQ087264 to JQ087274). Sequences showed 99.2 to 100% identity between *L. garvieae* strains, with the sequence JF7663366 differing by 1 to 2 nucleotides from all other sequences (accession numbers are listed in Table 1) and 2 recently submitted *L. garvieae* genomes (GenBank accession numbers AP009332 and AP009333). Low identity (65.2 to 65.4%) was found with the alpha/beta gene from *L. lactis* subsp. *lactis* (GenBank accession number CP002365) and *L. lactis* subsp. *cremoris* (GenBank accession number NC 008527.1). One of the nucleotide differences observed between JF7663366 and other *L. garvieae* strains occurred in the primer B3 region (Fig. 1).

In comparison, 16S rRNA sequences of *Lactococcus garvieae* show >90% identity with sequences of *L. lactis*. Therefore, the results suggest the alpha/beta fold family hydrolase gene may offer a more robust assay target in terms of specificity.

The region of the alpha/beta fold family hydrolase gene of *Lactococcus garvieae* (GenBank accession number JF7663366) amplified by the LAMP assay shared 99.2% sequence identity with *L. garvieae* (GenBank accession numbers AP009332 and AP009333), and 64.6 and 66.9% identity with *L. lactis* subsp. *lactis* (GenBank accession number CP002365)

and *L. lactis* subsp. *cremoris* (GenBank accession number NC 008527.1), respectively. Additionally, the alpha/beta fold family hydrolase amino acid sequence of *L. garvieae* (GenBank accession numbers JF76 6366) showed 98.5% identity with *L. garvieae* (GenBank accession numbers AP009332 and AP009333), and 64.1 and 64.6% identity with *L. lactis* subsp. *lactis* (GenBank accession number CP002365) and *L. lactis* subsp. *cremoris* (GenBank accession number NC008527.1), respectively.

Fig. 3. Optimization of reaction time for loop-mediated isothermal amplification (LAMP) reaction (30, 45 and 60 min). Lane M: 100 bp ladder used as a size marker; Lanes 1 to 7: *Lactococcus garvieae* genomic DNA. Lane 1: 100 ng; Lane 2: 10 ng; Lane 3: 1 ng; Lane 4: 100 pg; Lane 5: 10 pg; Lane 6: 1 pg; Lane 7: 100 fg

Optimized LAMP assay for *Lactococcus garvieae* detection

The optimum temperature for LAMP was found to be 60°C based on greatest clarity of the product bands at this temperature (Fig. 2). The amplification generated ladder-like pattern bands on the agarose gels, with sizes ranging from 180 bp up to the loading wells (Fig. 2). With respect to reaction time, no product amplification occurred at 15 min (data not shown). However, 30, 45 and 60 min amplification at 60°C formed ladder-like pattern bands at 100 ng DNA (Fig. 3). The limit of DNA detection of the

Fig. 2. Optimal reaction temperature over 60 min. incubation for loop-mediated isothermal amplification (LAMP) was determined based on inspection of amplification products on gels. Lane M: 100 bp DNA ladder; Lanes 1 to 8 show results for incubation at 65.0, 63.5, 61.7, 59.8, 57.9, 56.0, 54.1 and 52.2°C, respectively

LAMP assay was tested at 30, 45 and 60 min, and results showed the limit of DNA detection at 1 pg (reaction time for 60 min), 1 ng (45 min) and 100 ng (30 min), respectively (Fig. 3). The results of LAMP by visual inspection with diluted SYBR Green I agreed with those of gel electrophoresis (data not shown). Hence, the amplification conditions were optimized for 60 min at 60°C.

Specificity of LAMP

The species specificity of LAMP primers was evaluated using 30 *Lactococcus garvieae* strains and 15 non-*L. garvieae* strains (Table 1). All strains of *L. garvieae* were detected by LAMP (Table 1). No non-*L. garvieae* strains were amplified by the LAMP assay.

Detection limits of LAMP and PCR

The alpha/beta fold family hydrolase gene LAMP assay could detect purified DNA template at 2 pg per reaction in all triplicate reactions, although the pattern at 2 pg reaction was weaker compared to reactions with higher DNA concentrations (Fig. 4A). The results of LAMP by visual inspection with diluted SYBR Green I agreed with those of gel electrophoresis (Fig. 4C). The PCR using primers pLG-1 and pLG-2 amplified products with 20 pg purified DNA per reaction (Fig. 4B). With respect to crude lysate, LAMP could amplify *Lactococcus garvieae* DNA in reactions containing down to 3×10^2 CFU (Fig. 5A), while 16S rDNA PCR could amplify down to 3×10^4 CFU (Fig. 5B). Therefore,

Fig. 4. Comparison of sensitivity between the (A) loop-mediated isothermal amplification (LAMP) (60 min at 60°C) and (B) 16S rDNA PCR assays for detection of the *Lactococcus garvieae* purified genomic DNA. Detection of *L. garvieae* by LAMP reactions was determined based on both inspection of amplification products on gels and visual inspection of (C) SYBR Green I fluorescence. Lane M: 100 bp ladder used as a size marker; Lane 1: 200 ng DNA per reaction; Lane 2: 20 ng DNA per reaction; Lane 3: 2 ng DNA per reaction; Lane 4: 200 pg DNA per reaction; Lane 5: 20 pg DNA per reaction; Lane 6: 2 pg DNA per reaction; Lane 7: 200 fg DNA per reaction

Fig. 5. Comparison of sensitivity between the (A) loop-mediated isothermal amplification (LAMP) and (B) PCR assays for detection of the *Lactococcus garvieae* bacteria in crude lysates. Lane M: 100 bp ladder used as a size marker; Lane 1: 3 × 10⁶ colony-forming units (CFU); Lane 2: 3 × 10⁵ CFU; Lane 3: 3 × 10⁴ CFU; Lane 4: 3 × 10³ CFU; Lane 5: 3 × 10² CFU; Lane 6: 3 × 10¹ CFU; Lane 7: 3 CFU

Table 2. Comparative *Lactococcus garvieae* detection for polymerase chain reaction (PCR) (16S rDNA; Zlotkin et al. 1998) and loop-mediated isothermal amplification (LAMP) (alpha/beta fold family hydrolase gene) methods. DNA extracts from infected fish tissue used as template. PCR and LAMP reactions performed in triplicate for each fish; all triplicate reactions gave the same results. GM: grey mullet; T: tilapia; S: spleen; K: kidney; B: brain

Assay	Fish	Infected tissue (positive/total sample)								
		S1	S2	S3	K1	K2	K3	B1	B2	B3
LAMP	GM	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
	T	15/15	15/15	15/15	15/15	15/15	15/15	15/15	15/15	15/15
PCR	GM	5/5	5/5	5/5	5/5	5/5	4/5	5/5	5/5	5/5
	T	3/15	9/15	14/15	5/15	7/15	12/15	3/15	4/15	8/15

the sensitivity of the LAMP assay was determined to be 100-fold higher than that of PCR using crude bacterial lysate.

Comparative analysis in infected fish tissue detection

The presence of *Lactococcus garvieae* in *L. garvieae*-challenged tilapia and grey mullet was detected using the LAMP and PCR assays (Table 2). All grey mullet died and exhibited haemorrhage on the fins, opercula and body surface 24 h post-challenge with *L. garvieae*. By contrast, tilapias were not observed with marked clinical signs and were sacrificed 24 h post challenge. However, *L. garvieae* were reisolated from all infected fish and identified by PCR. *L. garvieae* was detected by the LAMP assay in all infected spleen, kidney and brain samples from grey mullet and tilapia (Table 2). No bacterial DNA was detected by PCR or LAMP assay in the negative controls. The 16S rDNA PCR assay detected *L. garvieae* in fewer infected fish samples compared to the LAMP assay (Table 2). The same results were obtained in triplicate analysis per sample with respect to both LAMP and PCR assays.

DISCUSSION

Lactococcosis incurs major economic losses in the aquaculture industry. In Taiwan, lactococcosis has appeared regularly in grey mullet farms from 2002, representing an important risk factor in the grey mullet industry during the summer (Chen et al. 2002). This disease has been found in Taiwan in other aquatic animals such as rainbow trout, giant freshwater prawns, tilapia and bullfrogs, necessitating the development of a rapid and sensitive detec-

tion method to help limit its further spread.

PCR is a widely used detection method for various fish pathogens. PCR-based assays for *Lactococcus garvieae* include amplification of the 16S ribosomal RNA gene (Zlotkin et al. 1998, Pu et al. 2002) and dihydropteroate synthase gene (Aoki et al. 2000). However, conventional PCR requires using expensive equipment and other costly materials; electrophoresis must also be per-

formed to detect the amplicon. The whole process can take several hours. A real time PCR employing a detection and quantification method based on the 16S rRNA gene for *L. garvieae* has also been established (Jung et al. 2010). The real time PCR is a superior method to traditional PCR in sensitivity because of the detection method used. It also allows starting target template to be quantified. The minimum level of detection by the real time PCR was 32 fg of purified genomic DNA, equivalent to approx. 10 *L. garvieae* cells (Jung et al. 2010). This method shows potentially greater sensitivity in the detection of *L. garvieae* compared to our LAMP assays, though this cannot be stated conclusively without direct comparison on the same material. However the requirements of high cost thermocyclers, fluorescent probes, dyes, etc. make this method very expensive. The rapidity, simplicity and sensitivity of LAMP makes it highly promising for detecting pathogens in aquaculture facilities and for managing diseases (Savan et al. 2005). LAMP reaction is performed under isothermal conditions and does not require expensive equipment or costly kits. A water bath or block heater is enough for DNA amplification. Additionally, LAMP method is very specific since it uses 4 primers that recognize a total of 6 specific sequences. The LAMP method also allows easy and rapid visualization of the amplified products without the need for gel electrophoresis. Visual inspection of LAMP amplification products using SYBR Green I required approximately 1 min to declare the sample positive or negative.

The optimum conditions of LAMP reaction to detect *Lactococcus garvieae* DNA were 60°C and 60 min. However, according to our results, detection can be performed within 30 min at 60°C; though these conditions are less sensitive. In addition, the results of visual inspection with SYBR Green I correlate with those of gel electrophoresis. This finding

suggests that *L. garvieae* can be detected in an extremely short time (<60 min) by LAMP.

The *Lactococcus garvieae* alpha/beta fold family hydrolase gene contained within clone flg08-6 showed 64% identity with amino acid sequence of *L. lactis* subsp. *cremoris* alpha/beta fold family hydrolase (accession number YP_808850). We observed that alpha/beta fold family hydrolase gene sequences from 12 *L. garvieae* strains were identical with only strain JF766366, differing from the others by 1 to 2 nucleotides. Therefore, the alpha/beta gene shows more divergence between closely related species compared to other genes targeted in previously developed assays but is largely conserved within the *L. garvieae* species, making it a good assay candidate for specificity and robustness. For these reasons, the alpha/beta fold family hydrolase gene was selected for the LAMP primer design.

The results indicate that LAMP provides high specificity for the detection of *Lactococcus garvieae*, successfully detecting different isolates of *L. garvieae* but not closely related bacterial species, some of which are important bacterial pathogens of fish. The PCR assay targeting the 16S rDNA, developed by Zlotkin et al. (1998), has been used to identify *L. garvieae* as described previously (Chen et al. 2002, Colorni et al. 2003, Kang et al. 2004, Wang et al. 2007, Madinabeitia et al. 2009). The present study compared the sensitivities of both PCR (Zlotkin et al. 1998) and LAMP. The endpoint detection limit for purified DNA for the LAMP assay was approximately 10 times more sensitive than that of the 16S rDNA conventional PCR. The detection limit of the LAMP assay was 2 pg of *L. garvieae* DNA, visualized by electrophoresis and visual inspection with SYBR Green I. A band was observed with 20 pg DNA or above by conventional PCR. Testing of *L. garvieae* in pure cultures revealed that the LAMP assay was 100 times more sensitive than the conventional PCR, capable of detecting 300 CFU of *L. garvieae* per reaction tube. This higher sensitivity of LAMP compared to PCR has been described elsewhere (Han & Ge 2008). Moreover, the greater sensitivity of LAMP with crude lysate might indicate that LAMP is more robust with respect to dirty samples, because the LAMP method uses a robust DNA polymerase with low sensitivity to inhibitors (Paris et al. 2007).

Sensitive and specific assays that can be performed efficiently on infected fish tissue in a clinical laboratory are essential for early detection of pathogens and appropriate and effective antibiotic therapy. Bacteria were isolated from spleen, kidney and brain of all experimentally infected tilapia and grey mullet

and identified as *Lactococcus garvieae* by PCR using pLG-1 and pLG-2. LAMP could detect *L. garvieae* in all infected fish tissue sampled. Detection of *L. garvieae* in infected grey mullet tissue by 16S rDNA PCR was observed to be almost as high as that of LAMP. However, in tilapia, the PCR method was not as sensitive as LAMP. Above results suggest that the LAMP assay developed here is more sensitive than the Zlotkin et al. (1998) PCR in terms of detecting *L. garvieae* in fish tissue. To our knowledge, this study describes for the first time the feasibility of applying the LAMP assay technique to rapidly detect *L. garvieae* in fish. This method is simple, rapid, specific and sensitive, and highly promising for detection of *L. garvieae* in clinical applications.

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