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Shewanella putrefaciens in cultured tilapia detected by a new calcein-loop-mediated isothermal amplification (Ca-LAMP) method

Rungkarn Suebsing[1], Jantana Kampeera[1], Sarawut Sirithammajak[1], Padmaja Jayaprasad Pradeep[3], Sarocha Jitrakorn[2,3], Narong Arunrut[1], Pakkakul Sangsuriya[2], Vanvimon Saksmerprome[2,3], Saengchan Senapin[2,3], Boonsirm Withyachumnarnkul[2,4,5], Wansika Kiatpathomchai[1,*]

[1]Bioengineering and Sensing Technology Laboratory, [2]BIOTEC, National Science and Technology Development Agency (NSTDA), 113 Thailand Science Park, Phahonyothin Rd., Klong Neung, Klong Luang, Pathum Thani 12120, Thailand

[3]CENTEX Shrimp, Faculty of Science, Mahidol University, Rama VI Rd., Bangkok 10400, Thailand

[4]Department of Anatomy, Faculty of Science, Mahidol University, Rama VI Rd., Bangkok 10400, Thailand

[5]Aquatic Animal Biotechnology Research Center, Faculty of Science and Industrial Technology, Prince of Songkla University, Suratthani Campus, Surat Thani 84100, Thailand

ABSTRACT: *Shewanella putrefaciens* is being increasingly isolated from a wide variety of sources and is pathogenic to many marine and freshwater fish. For better control of this pathogen, there is a need for the development of simple and inexpensive but highly specific, sensitive, and rapid detection methods suitable for application in field laboratories. Our colorogenic loop-mediated isothermal amplification (LAMP) assay combined with calcein (Ca-LAMP) for unaided visual confirmation of LAMP amplicons is a simple method for fish pathogen detection in cultured tilapia. Here, we describe the detection of *S. putrefaciens* using the same platform. As before, the method gave positive results (orange to green color change) in 45 min at 63°C with sensitivity 100 times higher than that of a conventional PCR assay, with no cross-amplification of other known fish bacterial pathogens tested. Using the assay with 389 samples of gonads, fertilized eggs, and fry of farmed Nile and red tilapia *Oreochromis* spp., 35% of samples were positive for *S. putrefaciens*. The highest prevalence was found in samples of gonads (55%) and fertilized eggs (55%) from adult breeding stocks, indicating that *S. putrefaciens* could be passed on easily to fry used for stocking production ponds. Tissue tropism assays revealed that the spleen showed the highest colonization by *S. putrefaciens* in naturally infected tilapia and that it would be the most suitable organ for screening and monitoring fish stocks for presence of the bacteria.

KEY WORDS: Colorimetric · LAMP · Calcein · *Oreochromis* spp. · Tilapia · Tissue tropism

INTRODUCTION

Tilapia *Oreochromis* spp. are among the most economically important freshwater fish in worldwide aquaculture. These fish are affected by several pathogens, including *Streptococcus* spp., *Flavobacterium columnare*, *Aeromonas* spp., and betanodavirus (Bigarré et al. 2009, Eissa et al. 2010, Li & Cai 2011, Suebsing et al. 2013). Recently, *Shewanella putrefaciens* has been reported to cause mortalities in a wide variety of freshwater and marine fish species, including tilapia (Kozinska & Pekala 2004, Korun et al. 2009, Lu & Levin 2010, Qin et al. 2012).

*Corresponding author: wansika@biotec.or.th

S. putrefaciens is a Gram-negative, rod-shaped, oxidative bacterium that produces hydrogen sulfide gas (H_2S) (Khashe & Janda 1998) and is a major cause of fish bacteremia and spoilage (Chiniwasagam et al. 1996, Vogel et al. 2005). It triggers autolysis of muscle cells and chemical oxidation of lipids (Dalgaard 1995, Gram & Huss 1996). It is commonly found in soil and water, and is considered to be an opportunistic human pathogen (Semple et al. 1989, Brink et al. 1995, Pagani et al. 2003). While the typical source of human infection is exposure of skin lesions or skin trauma in marine environments (Pagani et al. 2003), little is known about its mode of transmission in fish.

DNA analysis of bacterial isolates has revealed that traditional *S. putrefaciens* is genetically heterogeneous, consisting of 4 distinct genomic groups numbered I to IV (Owen et al. 1978). Group I is designated as true *S. putrefaciens* (Tryfinopoulou et al. 2007), Group II as *S. baltica* (Ziemke et al. 1998), Group III as *S. oneidensis* (Venkateswaran et al. 1999), and Group IV as *S. algae* (Simidu et al. 1990). It was found that *S. algae* was often misidentified as *S. putrefaciens* (Vogel et al. 1997), and that more than 80% of clinical isolates from humans are actually *S. algae*, while isolates from fish and environmental sources were *S. putrefaciens* (Holt et al. 1997, Vogel et al. 1997).

Molecular biology-based analysis such as polymerase chain reaction (PCR) and real-time PCR methods are recognized as powerful tools for detecting *S. putrefaciens* (DiChristina & DeLong 1993, Miller et al. 2010). However, the application of such technology is still limited due to the need for specialized instruments and trained individuals to carry out the tests and interpret the results. Development of a loop-mediated isothermal amplification (LAMP) assay combined with the fluorescent metal dye calcein (Ca-LAMP) for simple visual detection (Tomita et al. 2008) of *Streptococcus agalactiae* and *Streptococcus iniae* in tilapia overcame these limitations (Suebsing et al. 2013). Thus, we have developed a similar method for the detection of *S. putrefaciens* in tilapia and used it to study *S. putrefaciens* prevalence in fry, farmed broodstock, and rearing water.

MATERIALS AND METHODS

Sample collection and DNA preparation

For field evaluation using the newly developed Ca-LAMP assay for detection of *Shewanella putrefaciens*, we haphazardly collected Nile tilapia *Oreochromis niloticus* and a red tilapia hybrid (*O. niloticus* × *O. mossambicus*) from Nam Sai Farm, a commercial tilapia production facility in Prachinburi, Thailand. These samples included gonads of 40 male and 40 female broodstock (200–500 g body weight, BW) that were apparently healthy, as they showed no gross signs of bacterial infections and had been used in a routine breeding program. Approximately 50 mg of ovarian or testicular tissue was aseptically removed from cold-anesthetized specimens. Pools of fertilized eggs (n = 42; 10 eggs pool^{-1}) were gently taken from the mouths of a different set of 20 grossly healthy, arbitrarily selected brooders and transferred to clean, separate containers. Pooled samples (n = 237; 5 fry sample^{-1}) of newly, artificially hatched fry (~10 d old) were haphazardly collected from the hatchery of the same farm. Water samples (10 of 50 ml each) were collected from rearing ponds stocked with broodstock and filtered through a 0.45 µm filter (Millipore) to collect residue on the filter to test separately for the presence of *S. putrefaciens*. All sample types, apart from eggs, were stored in 200 µl of rapid extraction solution (see below) and in 200 µl phosphate-buffered saline (PBS, pH 7.0) at −20°C until use for DNA extraction and bacteria isolation, respectively. Eggs were stored in extraction solution only.

To determine the tissue tropism of *S. putrefaciens* in farmed tilapia, a separate set of randomly selected, grossly normal broodstock (200–500 g BW) of Nile tilapia (n = 3) and red tilapia (n = 10) were collected and cold-anesthetized. Whole blood samples obtained from the tail vein were spotted directly on ISOCODE® filter paper (Schleicher & Schuell) in 50 µl spots as previously described (Kiatpathomchai et al. 2004). Tissue (~50 mg each) from 6 organs (brain, gills, spleen, anterior kidney, liver, and sex organs) showing no clinical signs of disease were removed aseptically from each fish.

Total DNA was extracted from each individual sample and from 5 ml of bacterial culture from reference and farm isolates (see below) using a rapid extraction solution (0.025 N sodium hydroxide and 0.0125% sodium dodecyl sulfate) as previously reported, with supernatant clarified by centrifugation (Jaroenram et al. 2009). Extracted nucleic acid was then diluted 100×, and 2 µl of the resulting solution were used as the template for either the Ca-LAMP assay or traditional PCR assay.

Bacterial isolates

Seventeen known fish pathogens from culture collections were used to determine the specificity

of the colorogenic Ca-LAMP developed for *S. putrefaciens* (Table 1). Stock suspensions of all isolates were stored in 20% glycerol at –80°C. To prepare the bacteria for DNA extraction, they were grown in tryptic soy broth (TSB) at 30°C for 24 h with gentle shaking. Bacterial genomic DNA templates were extracted using the rapid extraction solution described above.

Optimization of colorogenic LAMP assay with pre-addition of calcein

To design LAMP primers (Table 2) for detection of traditional *S. putrefaciens* likely to infect fish (i.e. covering new Groups I to III), we used published sequences of the 16S rRNA gene of *S. putrefaciens* ATCC 8071 (Group I), *S. baltica* NCTC 10735 (Group II), and *S. oneidensis* ATCC 700550 (Group III) to identify consensus primer sites. The Ca-LAMP assay was performed in a total reaction mixture of 25 µl containing 2 µM each of FIP and BIP, 0.2 µM each of F3 and B3, 2 µM each of LF and LB primers, 1× thermopol-supplied reaction buffer, 0.6 M betaine (USB), 6 mM $MgSO_4$ (New England BioLabs), 1.2 mM dNTPs mix (Thermo Fisher Scientific), 8 U *Bst* DNA polymerase (New England BioLabs), 25 µM calcein (with 0.5 mM $MnCl_2$; Sigma-Aldrich), and 2 µl total DNA template from *S. putrefaciens* ATCC 8071. A reaction mixture without template was included as a negative control. To determine optimum temperature conditions, the Ca-LAMP reaction was run at 60, 63, and 65°C at a fixed time of 60 min and the reaction was stopped at 85°C for 5 min. After selection of 63°C as the optimum temperature, reaction times of 15, 30, 45, and 60 min were tested to determine the optimum time. Color change from orange to green was assessed visually by the unaided eye. For comparison, the LAMP-amplified products were also detected by fluorescence spectrophotometer (CaryEclipse; Agilent Technologies) and by gel electrophoresis.

Traditional PCR assay

Primers for the single-step PCR detection of *S. putrefaciens* targeted the 16S rRNA gene and are listed as primers F3 and B3 in Table 2. PCR was carried out in a 25 µl reaction mixture containing 1× PCR supplied reaction buffer, 0.2 mM dNTPs (Thermo Fisher Scientific), 1.5 mM $MgCl_2$ (Invitrogen), 2.5 U *Taq* polymerase (Invitrogen), 10 µM each forward and reverse primer, and 2 µl of total DNA extract from *S. putrefaciens* ATCC 8071 as the template. The amplification was conducted with an initial cycle at 95°C for 3 min followed by 35 cycles of 95°C for 20 s, 63°C for 20 s, and 72°C for 30 s. A final extension step was at 72°C for 3 min. The PCR amplicons were visualized by agarose gel electrophoresis.

Table 1. Bacterial strains used to test the specificity of the colorogenic loop-mediated isothermal amplification combined with calcein (Ca-LAMP) assay. ATCC: American Type Culture Collection; NCTC: National Collection of Type Cultures; DMST: Culture collection for Medical Microorganisms, Department of Medical Science, Ministry of Public Heath, Thailand

Species	Strain number	Tube/Lane no. in Fig. 2
Shewanella putrefaciens	ATCC 8071	1–5
Shewanella baltica	NCTC 10735	6
Shewanella oneidensis	ATCC 700550	7
Flavobacterium psychrophilum	ATCC 49418	8
Flavobacterium columnare	ATCC 49512	9
Flavobacterium johnsoniae	Laboratory strain	10
Streptococcus agalactiae	DMST 17129	11
Streptococcus iniae	ATCC 29178	12
Staphylococcus epidermidis	ATCC 12228	13
Aeromonas salmonicida	ATCC 14174	14
Aeromonas hydrophila	ATCC 35654	15
Aeromonas sobria	Laboratory strain	16
Aeromonas veronii	Laboratory strain	17
Lactococcus garviae	ATCC 49156	18
Edwardsiella tarda	ATCC 15947	19
Vibrio anguillarum	ATCC 19264	20
Pseudomonas aeruginosa	ATCC 27853	21

Table 2. Oligonucleotide primers used in the Ca-LAMP method for detection of *Shewanella putrefaciens.* F3: forward outer; B3: backward outer; FIP: forward inner; BIP: backward inner; LF: loop forward; LB: loop backward. TTTT linker for primers FIP and BIP in **bold**

Primer	Sequence (5′–3′)
She-F3	GCGAGGTGGAGCTAATCTCA
She-B3	CCACAAAGTGGTGAGCGC
She-FIP	CCGTGGCATTCTGATCCACGAT-**TTTT**-CGGATTG GAGTCTGCAACT
She-BIP	CGTTCCCGGGCCTTGTACAC-**TTTT**-CCCGAAGG TTAAGCTACCCA
She-LF	GCGATTCCGACTTCATGGAGTC
She-LB	CCCGTCACACCATGGGAGTG

Specificity of Ca-LAMP assay

Total DNA extracts from 17 known fish pathogens (Table 1) were subjected to the Ca-LAMP assay. Any resulting LAMP amplicons were confirmed by restriction enzyme digestion, after their purification using a gel purification kit (Macherey-Nagel) according to the manufacturer's protocol. The purified amplicons were digested using *Xmn*I restriction enzyme (New England BioLabs) at 37°C for 1 h followed by 3% agarose gel electrophoresis. An *Xmn*I enzyme digested fragment of 113 bp was predicted based on the predicted amplicon sequence of *S. putrefaciens* as determined using the BioEdit program (Hall 1999).

A selection of these samples positive by Ca-LAMP assay were subjected to sequencing analysis to confirm the target sequence. The amplified products were cut with an *Xmn*I restriction enzyme and the digested fragment was cloned into the pJET vector (Thermo Fisher Scientific). Plasmid DNA was subsequently purified using an extraction kit (Favorgen) and 50 ng μl^{-1} of plasmid DNA were then sequenced directly using vector primer (SP6 5′-ATT TAG GTG ACA CTA TAG-3′; Macrogen). The 16S rRNA gene sequences were aligned with published sequences available in the GenBank database (NCBI) using ClustalW and MEGA software version 4 (Tamura et al. 2007).

Detection limit of Ca-LAMP and traditional PCR assays

For testing detection limits, *S. putrefaciens* ATCC 8071 was employed as a reference strain. DNA was extracted from a bacterial suspension containing 2.4 × 10^7 CFU ml^{-1}, determined by the standard plate count method, and prepared in 10-fold serial dilutions containing 2.4 × 10^7 (neat) to 2.4 CFU ml^{-1}. Using these DNA templates, in triplicate reactions, the Ca-LAMP assay and traditional PCR assay were compared in terms of their detection limit in detecting *S. putrefaciens* ATCC 8071.

Detection limit of Ca-LAMP and traditional PCR assays with spiked tissue samples

We assessed the effect of background host DNA on the efficiency and sensitivity of the Ca-LAMP and traditional PCR assays. DNA from homogenates of 6 organs (brain, gills, spleen, anterior kidney, liver, and sex organs), verified as negative for *S. putrefaciens* by the Ca-LAMP and PCR assays, were used as diluent, at a concentration of 100 ng $reaction^{-1}$ for 10-fold serial dilutions of *S. putrefaciens* ATCC 8071 DNA, with 3 replicates tested for each *S. putrefaciens* dilution. The sensitivity was then compared between the purified bacterial DNA and in the presence of host DNA.

Isolation and confirmation of bacterial isolates by biochemical tests and sequencing

Tissue (50 mg) from broodstock and pooled fry positive for *S. putrefaciens* by Ca-LAMP, and residue on the filter of water samples from ponds containing such fish, were homogenized in 200 µl PBS. Homogenates (100 µl) were screened for bacteria by inoculating onto TSA supplemented with 1.5% NaCl for 24 to 36 h at 30°C (Holt et al. 2005). Presumptive identification of *S. putrefaciens* colonies was based on Gram-negative staining, a positive oxidase test, and a positive catalase test. Colonies of 4 such representative isolates were then subjected to biochemical characterization using the API 20E kit (BioMerieux) with incubation at 30°C for 24 to 36 h. Their identities were also confirmed by sequence analysis of PCR amplicons from their 16S rRNA gene sequence (Weisburg et al. 1991).

RESULTS

Ca-LAMP conditions for *Shewanella putrefaciens* detection

When the Ca-LAMP assay was tested at 60, 63, and 65°C for 60 min, the reaction temperature of 63°C was selected as the standard for all subsequent tests since it exhibited the strongest color change by the naked eye and the clearest ladder-like pattern by gel electrophoresis (Fig. 1A). After fixing the reaction temperature at 63°C, reaction times of 15, 30, 45, and 60 min were tested and showed that visualized color change could be seen as early as 30 min, but that the most intense green color was observed at 45 min, which was identical to results using LAMP followed by gel electrophoresis (Fig. 1B). Thus, the optimal conditions for all subsequent Ca-LAMP reactions were set at 63°C for 45 min.

Specificity of Ca-LAMP assay

When the Ca-LAMP assay was tested for cross amplification with DNA templates from several fish

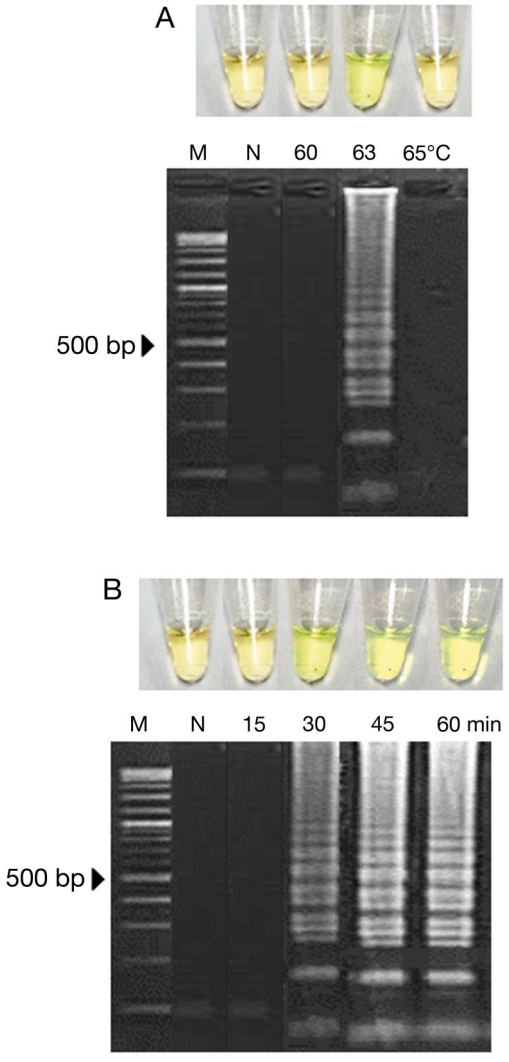

Fig. 1. Optimal conditions for the colorogenic loop-mediated isothermal amplification combined with calcein (Ca-LAMP) reaction. (A) Reaction temperature was held at 60, 63, and 65°C for 60 min. Based on the strongest color change and ladder-like pattern on the gel, 63°C was selected as the standard temperature for subsequent tests. (B) Reaction times of 15, 30, 45, and 60 min were tested at 63°C. Based on the results, 45 min was selected as the optimal time. Lane M: 2 log DNA marker; Lane N: negative control

bacterial pathogens (Table 1), all isolates in the traditional *S. putrefaciens* grouping (i.e. including *S. baltica* NCTC 10735 and *S. oneidensis* ATCC 700550 in Groups II and III as described in 'Materials and methods') gave positive test results, while no other tested bacteria were positive. This result indicated that our new Ca-LAMP assay was specific for traditional *S. putrefaciens* Groups I to III when tested against non-*Shewanella* genera (Fig. 2). *S. algae* (Group IV) was not available for testing, as it is absent from Thailand and importation of isolates is not allowed.

Digestion of the LAMP-positive amplicons with the restriction enzyme *Xmn*I followed by agarose gel electrophoresis yielded the expected fragment of 113 bp with simultaneous loss of the typical ladder-like electrophoresis pattern of the LAMP method (Fig. 3), confirming that the LAMP amplicons arose from the targeted 16S region of the rRNA gene of *S. putrefaciens*-specific primers.

Comparison of sensitivity between Ca-LAMP and PCR assays

In comparing the sensitivity of Ca-LAMP to a traditional PCR assay using DNA template extracted from various concentrations of *S. putrefaciens* ATCC 8071 (2.4×10^7 to 2.4 CFU), Ca-LAMP had a detection limit of 2.4 CFU (Fig. 2A,B), while that of the PCR assay was 2.4×10^3 CFU (Fig. 2B). The sensitivity of Ca-LAMP also gave identical results when amplicon amplification was detected by gel electrophoresis or by fluorescence spectrophotometry (Fig. 2B,C).

Effect of background host DNA

The effect of host DNA on sensitivity was compared using a 10-fold dilution series of genomic *S. putrefaciens* DNA in fish DNA. The presence of background host DNA (brain, gills, spleen, anterior kidney, liver, and sex organs) did not decrease the efficiency of the Ca-LAMP and PCR assays when compared with the sensitivity of pure culture (Fig. 4). In spiked samples (irrespective of the tissue type used as matrix), the sensitivity of Ca-LAMP and PCR was still 2.4 and 2.4×10^3 CFU, respectively (data not shown).

S. putrefaciens in farmed tilapia

The results for detection of *S. putrefaciens* in farmed tilapia are summarized in Table 3. A portion of all sample types (gonads, fertilized eggs, and fry) were found to be positive by both Ca-LAMP and PCR assays. Among 389 samples, 136 (34.96%) gave positive test results for *S. putrefaciens* by Ca-LAMP and were confirmed by sequence analysis, while the prevalence was 12.59% (49/389) by conventional PCR assay. The Ca-LAMP-positive samples included 44 gonad tissue samples, 24 fertilized-egg samples, and 68 fry samples. All PCR-positive samples were

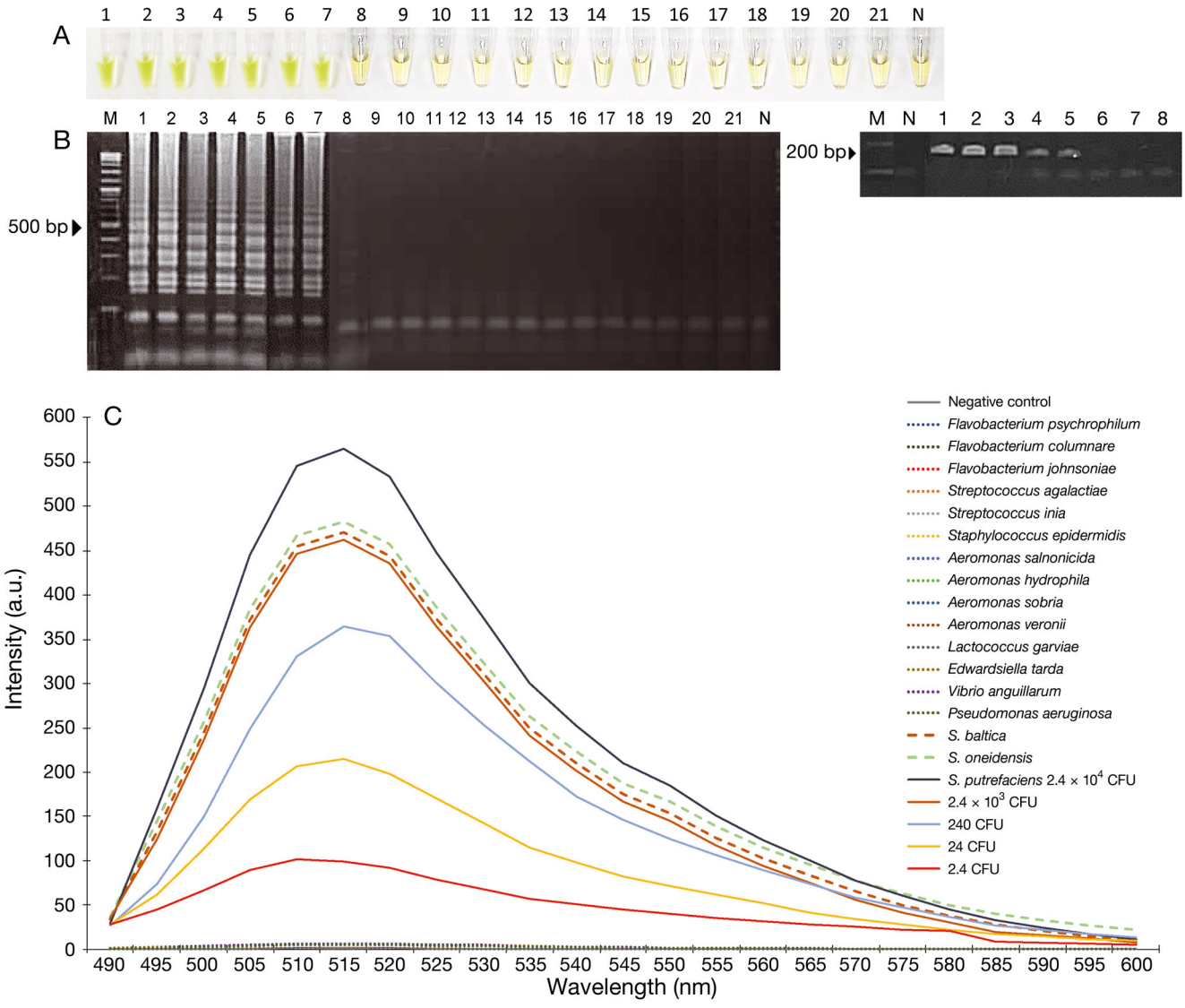

Fig. 2. Sensitivity and specificity of the Ca-LAMP assay for detecting *Shewanella putrefaciens* with (A) pre-added calcein, (B) followed by gel electrophoresis (left panel: LAMP; right panel: PCR). Tube numbers in (A) correspond to Lane numbers in (B): (1–5) *S. putrefaciens* ATCC 8071 at 10-fold dilutions from 2.4×10^4 to 2.4 CFU; (6–21) details for other strains are listed in Table 1. Lane M: 2 log DNA marker; Lane N: negative control. (C) Fluorescence spectra analysis of colorogenic LAMP sensitivity (excitation at 480 nm) corresponding to Tubes 1–21

also Ca-LAMP positive. In addition, 8 of 10 water samples taken from ponds where the positive broodstock were reared also gave positive test results for *S. putrefaciens* by the Ca-LAMP assay, whereas 3 water samples were *S. putrefaciens*-positive by conventional PCR assay.

S. putrefaciens tissue tropism analysis of 7 different tissues from 13 farmed, grossly healthy broodstock tilapia revealed that 11 of 13 were positive for *S. putrefaciens* by the Ca-LAMP assay. With respect to test results for various tissues of the 11 positive tilapia, spleen tissue was the most frequently positive (61.54%), followed by gills (46.15%), kidney (30.77%), blood (23.08%), brain (23.08%), liver (23.08%), and sex organs (15.38%; Table 4). In contrast to Ca-LAMP, only 4 of 13 fish gave positive results using normal PCR assays, probably due to the low analytical sensitivity of the assay (Table 4).

S. putrefaciens phenotype characteristics

Biochemical characteristics of *S. putrefaciens* isolated from tilapia and water samples were compared

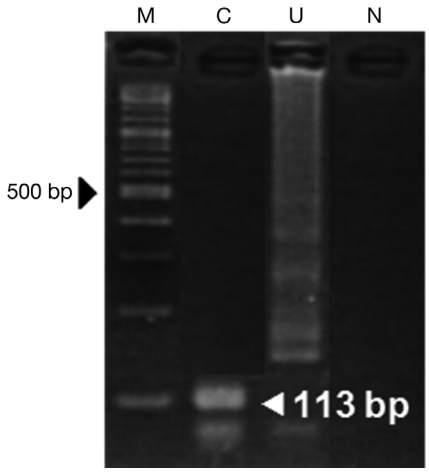

Fig. 3. Confirmation of amplified Ca-LAMP products by restriction enzyme analysis. Lane M: 2 log DNA marker; Lane N: negative control; Lane C: *Xmn*I enzyme digested fragment of 113 bp; Lane U: uncut LAMP product

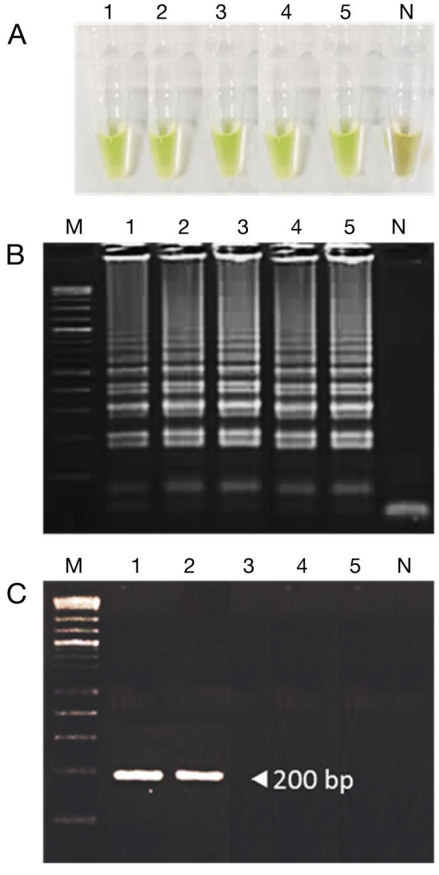

Fig. 4. Sensitivity comparison of Ca-LAMP (A) by the naked eye and (B) by gel electrophoresis. (C) PCR assay for *Shewanella putrefaciens* ATCC 8071 detection in the presence of background DNA. Tube numbers in (A) correspond to Lane numbers in (B) and (C): (1–5) *S. putrefaciens* ATCC 8071 at 10-fold dilutions from 2.4×10^4 to 2.4 CFU; N: negative control; Lane M: 2 log DNA marker

Table 3. Comparison of the prevalence of bacteria in *Shewanella putrefaciens* Groups I to III in farmed tilapia *Oreochromis* spp. as determined by the Ca-LAMP and PCR assays

Type of sample	n	Detection, n (%) LAMP	PCR
Nile tilapia *O. niloticus*			
Broodstock:			
Female (ovary)	20	10 (50.00)	4 (20.00)
Male (testis)	20	15 (75.00)	10 (50.00)
Fertilized egg[a]	22	22 (100.00)	1 (4.55)
Fry[b]	131	13 (9.92)	6 (4.58)
Red tilapia *Oreochromis* sp.			
Broodstock:			
Female (ovary)	20	9 (45.00)	7 (35.00)
Male (testis)	20	10 (50.00)	6 (30.00)
Fertilized egg[a]	21	2 (9.52)	0 (0.00)
Fry[b]	136	55 (40.44)	15 (11.03)
Total	**389**	**136 (34.96)**	**49 (12.59)**

[a]10 eggs pooled per sample
[b]5 fry pooled per sample

to reference strains (Table 5). Representative isolates (n = 4) showed biochemical characteristics similar to *S. putrefaciens* Groups I to VI (Ziemke et al. 1998, Holt et al. 2005), including H_2S production, citrate utilization, weak hemolysis on blood agar after incubation for 48 h, glucose and arabinose utilization, and growth in high-salt solution. When these 4 isolates were subjected to 16S rRNA gene sequencing (GenBank accession numbers KF850533–KF850536) and aligned with sequences of *Shewanella* entries in the GenBank database, they were found to share 97 to 99% sequence identity with traditional *S. putrefaciens* Group I (data not shown).

DISCUSSION

LAMP has well documented advantages for the detection of human and aquatic animal pathogens. These include rapid diagnosis with a high sensitivity and specificity equivalent or superior to more conventional methods (Savan et al. 2005, Mori & Notomi 2009). Suebsing et al. (2013) successfully developed the Ca-LAMP method to detect *Streptococcus agalactiae* and *S. iniae* in farmed tilapia and showed that its sensitivity and specificity were higher than those of a traditional PCR assay. Therefore, we developed a similar Ca-LAMP assay for direct screening of the presence of *Shewanella putrefaciens* in farmed tilapia without requirements for a secondary visualization step. We also com-

Table 4. Comparison of tissue tropism of *Shewanella putrefaciens* in naturally infected tilapia using the Ca-LAMP and PCR assays. M: male; F: female; √: positive result; x: negative result

Fish		Blood		Brain		Liver		Spleen		Kidney		Gills		Gonad	
Sex	ID	LAMP	PCR	LAMP	PCR	LAMP	PCR	LAMP	PCR	LAMP	PCR	LAMP	PCR	LAMP	PCR
Nile tilapia *Oreochromis niloticus*															
M	1	√	x	x	x	x	x	x	x	x	x	x	x	x	x
M	2	x	x	x	x	x	x	x	x	x	x	x	x	x	x
F	3	x	x	x	x	x	x	√	x	√	√	x	x	x	x
Red tilapia *Oreochromis* sp.															
M	1	√	√	√	x	√	√	√	x	x	x	√	x	x	x
M	2	x	x	x	x	x	x	x	x	x	x	x	x	x	x
M	3	√	√	√	√	√	√	√	√	√	√	√	√	√	x
M	4	x	√	x	x	√	√	√	√	√	√	√	√	√	√
M	5	x	x	√	x	x	x	√	x	x	x	x	x	x	x
F	6	x	x	x	x	x	x	x	x	x	x	x	x	x	x
F	7	x	x	x	x	x	x	√	x	√	x	x	x	x	x
F	8	x	x	x	x	x	x	√	x	x	x	√	x	x	x
F	9	x	x	x	x	x	x	√	x	x	x	√	x	x	x
F	10	x	x	x	x	x	x	x	x	x	x	√	x	x	x
Total	13	3/13	3/13	3/13	1/13	3/13	3/13	8/13	2/13	4/13	3/13	6/13	2/13	2/13	1/13
(%)		23.08	23.08	23.08	7.68	23.08	23.08	61.54	15.38	30.77	23.08	46.15	15.38	15.38%	7.68

pared the Ca-LAMP assay with a more conventional PCR assay.

A LAMP assay was previously developed for detection of *S. putrefaciens* mRNA in preserved fish samples (Li et al. 2012). However, the *S. putrefaciens*-LAMP primers in that study were not tested against all *S. putrefaciens* groups reported to cause mortalities in fish. In addition, visualization of the LAMP amplicons in that study was achieved by gel electrophoresis with ethidium bromide staining. Since *S. putrefaciens* is recognized as a genetically heterogeneous species, to understand the prevalence and tissue tropism of *S. putrefaciens* in fish, our specific LAMP-primers were designed based on consensus sequences of 16S rRNA genes and could detect Groups I to III of *S. putrefaciens.* It is still unclear whether the LAMP primers in the present study can detect Group IV (*S. algae*), mostly isolated from human infected skin, because it was not tested. In addition, we re-checked and confirmed our BLASTN results from the GenBank database and also used ClustalW to compare the target region of our LAMP-primers with the matching regions of the 16S rRNA sequences of *Shewanella* groups (I to VI) in the database. The results revealed binding with all groups. While the assay does not inform directly on which *S. putrefaciens* group (I to III) is present, it allows the presence of any of Groups I to III to be detected, thus reducing the need for multiple assays and reactions in a screening program. Amplicons from positive samples can be subsequently sequenced if identification to group level is required.

The detection limit of Ca-LAMP was 2.4 CFU in pure culture, which was 1000 times higher that of traditional PCR (2.4×10^3 CFU) and the efficiency was not reduced in the presence of background DNA from different tissues, indicating that the Ca-LAMP was not affected by the potential presence of inhibitory substances in different tissue matrices. These observations indicate that the Ca-LAMP assay is very useful for the detection of *S. putrefaciens*. Although the phenotypic characteristics of the strain isolated from our study was verified based on the key characteristics of *S. putrefaciens* group (Ziemke et al. 1998, Holt et al. 2005), they did not provide a clear phenotypic indication to which group they belong. A reflection of the phenotypic diversity within *S. putrefaciens* has been reported previously (Khashe & Janda 1998). We also found a new phenotypic differential characteristic, including arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, and urease production, in this study. However, following DNA homology results, the 16S rRNA gene sequence generated high similarity (>97%) to *S. putrefaciens*. Thus, these results might confirm the high heterogeneity of the *Shewanella* cluster, and new isolates obtained from tilapia could be a different type of strain that have diverged from other *S. putrefaciens* isolates (Tryfinopoulou et al. 2007).

The Ca-LAMP screening for *S. putrefaciens* in farmed tilapia revealed 35% (136/389) prevalence, indicating that it may be a major source of bacterial infections in tilapia (Suebsing et al. 2013). Of particu-

Table 5. Physical and biochemical characteristics of *Shewanella putrefaciens* isolated from healthy tilapia, compared to previously identified *Shewanella* groups; all strains are Gram-negative rods, forming pale yellow colonies. TB53: isolated from Nile tilapia *Oreochromis niloticus* female broodstock; TG53: isolated from red tilapia *Oreochromis* sp. male broodstock; NG73: isolated from Nile tilapia fry; TB33: isolated from red tilapia fry. Strains used to represent each group are ATCC 8071 (Group I), NCTC 10735 (Group II), LMG 2266 (Group III), and *S. algae* LMG 2265 (Group IV) (Holt et al. 1997, 2005, Ziemke et al. 1998)

Characteristics	Sample				*S. putrefaciens* group			
	TB53	TG53	NG73	TB33	I	II	III	IV
Hemolysis on blood agar	β[a]	β[a]	β[a]	β[a]	–	β[a]	α[a]	β
Gliding motility	+	+	+	+	+	+	+	+
Production of:								
Catalase	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+
Nitrate reduction	–	–	–	–	–	–	–	+
β-galactosidase	+	+	+	+	+	+	+	+
Arginine dihydrolase	+	+	+	+	–	–	–	–
Lysine decarboxylase	+	+	+	+	–	–	–	–
Ornithine decarboxylase	+	+	+	+	–	–	–	–
Urease production	+	+	+	+	–	–	–	–
Tryptophane deaminase	–	–	–	–	–	–	–	–
Indole	–	–	–	–	–	–	–	–
Voges-Proskauer reaction	–	–	–	–	–	–	–	–
H_2S production	+	+	+	+	+	+	+	+
Citrate utilization	+	+	+	+	–	+	–	–
Acid production from:								
Glucose	+	+	+	+	+	+	+	(+)[a]
Mannitol	(+)[a]	(+)[a]	(+)[a]	(+)[a]	–	–	–	–
Sorbitol	–	–	–	–	–	–	–	–
Rhamnose	–	–	–	–	–	–	–	–
Sucrose	+	+	+	+	–	+	–	–
Melibiose	+	+	+	+	–	–	–	–
Amygdalin	+	+	+	+	–	–	–	–
Arabinose	+	+	+	+	+	–	–	–

[a]Weak reaction

lar importance was the presence of *S. putrefaciens* in gonads of healthy broodstock, in fertilized eggs, and in newly hatched fry, which therefore represents a potential reservoir for *S. putrefaciens* persistence or spreading under appropriate conditions. The potential for vertical transmission should be investigated. We suggest using the Ca-LAMP assay to screen broodstock before breeding and the newly hatched fry before rearing in ponds, in order to limit and control the bacterial infections. Water samples taken from rearing ponds with infected fish also contained *S. putrefaciens.* Further studies would be required to demonstrate whether levels of *S. putrefaciens* detected in water are a result of shedding from infected fish, or whether they represent background levels of *Shewanella* spp. in the environment. This is important for understanding the extent to which *S. putrefaciens* Groups I to III can be shed and spread from infected fish. *S. putrefaciens* is increasingly being found in diseased freshwater fish exhibiting 33 to 50% mortality (Kozinska & Pekala 2004, Lu & Levin 2010, Qin et al. 2012). It has been proposed that *Shewanella* spp. may have originated from the coastal marine environment and moved into the freshwater environment where they may now be considered a part of the normal fish microflora (Austin & Austin 1999).

S. putrefaciens infection was present in all tested organs of the cultured tilapia examined, indicating that it has broad tissue tropism and can infect many types of cells and tissues. Understanding this could assist us in studies on susceptibility and transmission of the disease, particularly for carriers, and might be useful in devising monitoring and control measures. Understanding tissue tropism of shrimp viruses (i.e. white spot syndrome virus, yellow head virus, and *Macrobrachium rosenbergii* nodavirus) has been useful in developing new detection protocols and investigating transmission routes (Lu et al. 1995, Lo et al. 1997, Sahul Hameed et al. 2004). We showed that *S. putrefaciens* was detected most frequently in the spleen of naturally infected tilapia, indicating that it would be the best target tissue to use in screening for *S. putrefaciens* in fish.

Finally, the Ca-LAMP detection is a time-saving and inexpensive method when compared with conventional bacterial culture and PCR methods. Using it to discover a relatively high prevalence of *S. putrefaciens* in tilapia reproductive organs will be important in studies on the mode of *Shewanella* transmission and control in tilapia, particularly in selective breeding programs aimed at developing specific pathogen-free stocks.

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