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Development of Loop-Mediated Isothermal Amplification (LAMP) Method for Rapid Detection of *Vibrio parahaemolyticus*

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Abstract

A technique for detecting *Vibrio parahaemolyticus* using a novel DNA amplification procedure designated loop-mediated isothermal amplification (LAMP) has been developed for the first time. A set of four primers, two outer and two inner primers, was designed specifically to recognize the thermolabile hemolysin gene (*tlh*) of *V. parahaemolyticus*. The LAMP reaction mix was optimized. The most optimal reaction temperature and time of the LAMP assay for the *tlh* gene was 60^{0}C and 60 min. Genomic DNAs from 28 bacterial strains including 14 *V. parahaemolyticus* strains were amplified using LAMP, and LAMP product was observed in other bacterial strains. The detection limit of this LAMP assay was approximately 90 fg. test tube $^{-1}$ of *V. parahaemolyticus* genomic DNA and 24 cfu.mL^{-1} for pure cultures. In addition, this method was applied to detect noncultured artificially contaminated food samples. These results suggest that detection of *V. parahaemolyticus* by the LAMP assay is an effective and low-cost procedure with high specificity and sensitivity that requires no specialized equipment. This assay is expected to become a valuable tool for rapid detection and identification of *V. parahaemolyticus*.

Introduction

Vibrio parahaemolyticus is a gram-negative, halophilic bacterium that distributes worldwide in the estuarine and coastal environment, especially in fishes, shellfishes and seafood products. It has been considered as one of the most important pathogens for both human and aquacultured animals. This pathogen is a common cause of foodborne illnesses in many Asian countries, including China and Japan, and is recognized as the leading cause of human gastroenteritis associated with seafood consumption in the United States (Su and Liu 2007). Nowadays, in China, the occurrence of the food poisoning caused by *V. parahaemolyticus* has increased remarkably, and this bacterium has become the leading foodborne pathogen of our country (Liu 2004). Thus, early and accurate detection of the pathogen is necessary for disease control.

Traditional cultivation methods including isolation and biochemical speciation

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is the primary tool for detection and identification of *V. parahaemolyticus*. These procedures are sophisticated and time-consuming generally taking at least 72 h. Recently, with the development of molecular biology, some powerful PCR-based techniques have been used for rapid detection of *V. parahaemolyticus* and the application for rapid diagnosis seems to be promising (Li et al. 2004; Ward and Bej 2006). These PCR-based methods are highly sensitive and specific, however, they are high-cost requiring sophisticated instruments for amplification and time-consuming because these methods usually require thermal cycling. Those methods are not in favor of rapid detection and diagnosis of this pathogen especially, for the on-the-spot test. Thus, a rapid, simple and cost-effective assay is needed to complement the current methods.

Notomi et al. (2000) recently reported a novel nucleic acid amplification method, designated loop-mediated isothermal amplification (LAMP), which amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions. LAMP relies on autocycling strand displacement DNA synthesis performed by a DNA polymerase with high strand displacement activity and a set of four specially designed primers (two inner and two outer primers). In the initial steps of the reaction, a stem-loop DNA structure is constructed as the starting material. In the later steps, one inner primer hybridizes to the loop on the LAMP product and initiates strand displacement DNA synthesis. This cycling reaction continues with accumulation of 10^9 copies of target within one hour (Notomi et al. 2000). Since it was first published in 2000, the LAMP method has been widely used in many fields, such as the rapid diagnosis of infectious diseases in clinic (Endo et al. 2004), qualitative and quantitative detection of some epidemic viruses (Imai et al. 2006) or bacteria (Hara-Kudo et al. 2005; Song et al. 2005; Yeh et al. 2005) and embryo sexing (Hirayama et al. 2006).

In the present study, we applied this recently developed loop mediated isothermal amplification method for the rapid detection of *V. parahaemolyticus* for the first time. LAMP specific primers were designed targeting the thermolabile hemolysin gene (*tlh*) of *V. parahaemolyticus*; the conditions of the assay were optimized; the specificity and sensitivity of the primers in the LAMP assay for detection of *V. parahaemolyticus* were determined and finally, the assay was applied to detect the pathogen in artificially contaminated food samples directly.

Materials and methods

Bacterial strains

Bacteria used in this study are listed in Table 1. The strains were from Institute of Microbiology of Chinese Academy of Science, Guangdong Institute of Microbiology in China, or isolated by our labs.

Table 1 List of bacterial strains used in this study and specificity of the LAMP primers

Bacterial species/strain	Medium	Growth temperature ^{0}C	LAMP
V. parahaemolyticus ATCC 33846	TCBS agar	30 ^{0}C	+
V. parahaemolyticus ATCC 33847	TCBS agar	30 ^{0}C	+
V. parahaemolyticus ATCC 17802	TCBS agar	37 ^{0}C	+
V. parahaemolyticus xq 1	TCBS agar	30 ^{0}C	+
V. parahaemolyticus xq 2	TCBS agar	30 ^{0}C	+
V. parahaemolyticus xq 3	TCBS agar	30 ^{0}C	+
V. parahaemolyticus xq 4	TCBS agar	30 ^{0}C	+
V. parahaemolyticus xq 5	TCBS agar	30 ^{0}C	+
V. parahaemolyticus xq 6	TCBS agar	30 ^{0}C	+
V. parahaemolyticus xq 7	TCBS agar	30 ^{0}C	+
V. parahaemolyticus xq 8	TCBS agar	30 ^{0}C	+
V. parahaemolyticus xq 9	TCBS agar	30 ^{0}C	+
V. parahaemolyticus xq 10	TCBS agar	30 ^{0}C	+
V. parahaemolyticus xq 11	TCBS agar	30 ^{0}C	+
Vibrio campbellii ATCC 33864	TCBS agar	30 ^{0}C	—
Vibrio fluvialis ATCC 33809	TCBS agar	30 ^{0}C	—
Vibrio harveyi ATCC 33842	TCBS agar	30 ^{0}C	—
Staphylococcus aureus subsp. aureus 1.1361	Nutrient agar	37 ^{0}C	—
Salmonella sp. 1.1552	Nutrient agar	37 ^{0}C	—
Listeria innocua GIM 1.230	Nutrient agar	37 ^{0}C	—
Listeria welshimeri GIM 1.231	Nutrient agar	37 ^{0}C	—
Listeria monocytohenes GIM 1.228	Nutrient agar	37 ^{0}C	—
Listeria monocytohenes GIM 1.229	Nutrient agar	37 ^{0}C	—
Vibrio cholerae zhy1	TCBS agar	30 ^{0}C	—
Vibrio cholerae zhy2	TCBS agar	30 ^{0}C	—
Vibrio cholerae zhy3	TCBS agar	30 ^{0}C	—
Escherichia coli	Nutrient agar	37 ^{0}C	—
Shigella	Nutrient agar	37 ^{0}C	—

Template DNA preparation

One-milliliter bacterial suspension was centrifuged at 12000 rpm for five minutes, and the supernatant was discarded. Pellets suspended in 100 ì l of sterile double water were boiled at 100^{0}C for ten minutes and immediate ice incubated for two minutes. After further centrifugation at 12000 rpm for five minutes, then supernatant was used as template DNA.

Design of LAMP primers

A set of species-specific LAMP primers comprising two outer and two inner primers was designed based on the highly conserved fragment of the *tlh* gene of *V. parahaemolyticus* (GenBank accession number M36437). The two outer primers were designated F3 and B3. The two inner primers were designated FIP and BIP. The FIP consisted of the F1c sequence (complementary to F1), a TTTT spacer and the F2 sequence. The BIP consisted of the B1c sequence (complementary to B1), a TTTT spacer and the B2 sequence. Primer sequences are shown in Table 2. Nucleotide sequences of targets for LAMP primers are illustrated in Fig 1.

Table 2. Sequence of LAMP primers for specific amplification of the *tlh* gene (GenBank accession no M36437)

Name	Sequence	Location
FIP	5'-GCCCATTCCCAATCGGTCG -TTTT-CTATGTTTCGC TGTTGGTATCG -3'	(323~ 305 ~ (258~ 27
BIP	5'-GTTCTACACCAACACGTCGCA-TTTT-TCGCCAAATCTAATGTTGCTTC -3'	(393~ 413 ~ (457~ 43
F3	5'-CAGCACGCAAGAAAACCA-3'	(231~ 248
B3	5'-ATTGTCAGCGGCGAAGAA -3'	(495~ 478

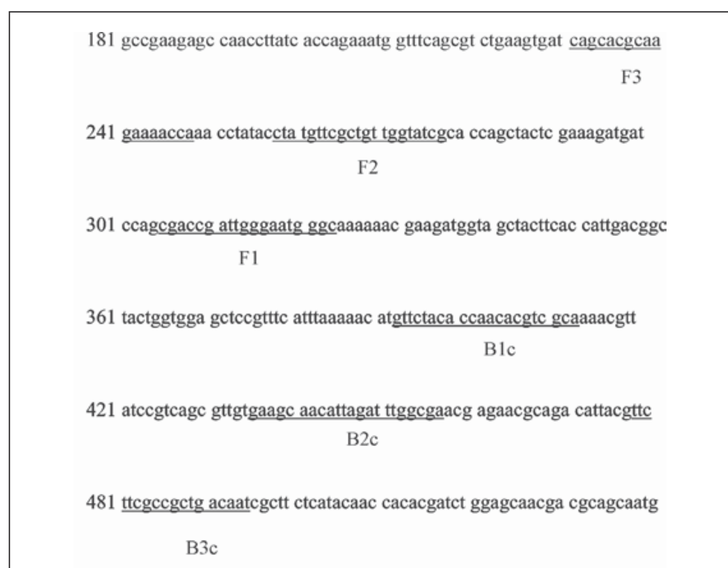

Figure 1. Nucleotide sequences of targets for LAMP primers on the *tlh* gene of *V. parahaemolyticus*.

LAMP reaction

The LAMP reaction was carried out in a 25 µl reaction mixture containing the following reagents with final concentrations: 8 mM $MgSO_4$, 1.0 mM dNTP, 0.8 M betaine (Sigma), 1.2 µM each of FIP and BIP primers, 0.2 µM each of F3 and B3 primers, 1 U *Bst*

DNA polymerase large fragment (New England Biolabs), 1x ThermoPol buffer and appropriate amount of template DNA. The reaction was carried out at 60^0C for one hour and inactivated at 80^0C for ten minutes (Notomi et al. 2000). Aliquots of 3 µl products were analyzed by 2 % agarose gel electrophoresis and detected under UV light after ethidium bromide staining. The change in turbidity of the reaction mixture was, meanwhile, observed by naked eyes.

Specificity of LAMP primers

The specificity of the set of LAMP primers for the *tlh* gene of *V. parahaemolyticus* was determined by LAMP amplification of the genomic DNAs from 14 *V. parahaemolyticus* and 14 bacterial strains other than *V. parahaemolyticus* listed in Table 1.

Sensitivity of LAMP assay

The sensitivity of the assay was determined using genomic DNAs and pure cultures of *V. parahaemolyticus* standard strains. Genomic DNA from *V. parahaemolyticus* ATCC 33846 was extracted and 10-fold serially diluted in sterile double water. Aliquots of 2 µl each dilution were amplified by LAMP using the optimum reaction condition. A LAMP reaction mixture containing no template DNA was used as a negative control.

To determine the sensitivity of detection for pure cultures, overnight cultivated *V. parahaemolyticus* ATCC 33846 was quantitated by direct plating and 10-fold serially diluted. Aliquots of 1 mL each dilution was used to prepare template DNA and 2 µl template DNA of each dilution were amplified by LAMP using the optimum reaction condition. A LAMP reaction mixture containing no template DNA was used as a negative control.

Detection of V. parahaemolyticus in artificially contaminated food samples

Shrimp samples were purchased from a local seafood store. 10 g of shrimp meat was added into 90 ml APW (peptone 10 g, sodium chloride 10 g, distilled water 1000 ml, pH 8.4), and homogenized in homogenizer for approimately 60 seconds to obtain the shrimp homogenate. Overnight cultivated *V. parahaemolyticus* ATCC 33846 suspensions (1 mL) were added into 10 mL shrimp homogenate to obtain artificially contaminated food samples. 1 mL of shrimp homogenate was used to prepare template DNA using Bacterial Genomic DNA Extraction Kit (Shanghai Sangon Biological Engineering Technology & Service CO., Ltd., China). LAMP amplification was performed under the optimum reaction condition. Template DNA extracted from uncontaminated shrimp homogenate was used as negative control.

Results

Standardization and optimization of LAMP assay

We initially standardized and optimized the LAMP assay for *V. parahaemolyticus*

detection using two outer and two inner primers from *tlh* gene and template DNAs from *V. parahaemolyticus* ATCC 33846 and ATCC 33847. The specific amplification generated the ladder-like pattern of bands (LAMP products) on agarose gel, and no band was observed in negative control (Fig. 2a). Besides, observing the LAMP reaction tubes by naked eyes, there was an increase in the turbidity of the reaction mixture with *V. parahaemolyticus* genomic DNA as the template DNA and no change was observed in the negative control tube; Centrifuged at 5000 rpm for several seconds, only the reaction tube with *V. parahaemolyticus* genomic DNA as the template DNA had white precipitate accumulating at the bottom of the tube (Fig. 2b).

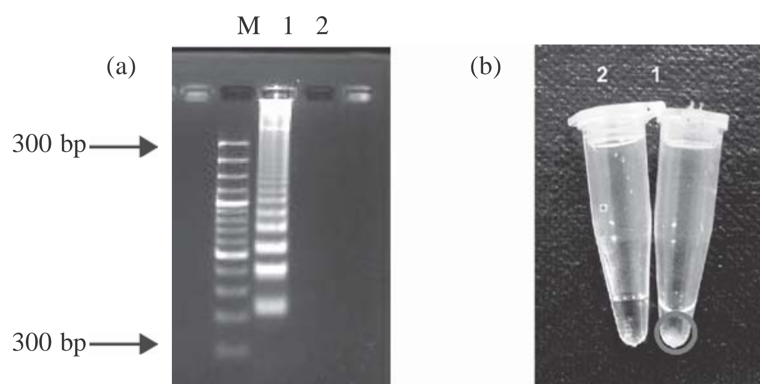

Figure 2. The LAMP result of *V. parahaemolyticus* standard strain.
M: 100 bp marker; 1: *V. parahaemolyticus* ATCC 33846; 2: negative control.

Effect of Mg^{2+} concentration

The LAMP reaction in the presence of various concentrations of Mg^{2+} was tested. We varied the Mg^{2+} concentrations from 2 to 18 mM to amplify the target DNA. Mg^{2+} concentration at 8 mM gave the maximal reaction product.

Effect of dNTPs concentration

The effect of dNTPs concentrations ranging from 0 to 1.8 mM on the LAMP reaction was tested. The results indicated that the optimal dNTPs concentration was 1.0 mM.

Effect of betaine concentration

The LAMP reaction in the presence of different concentrations of betaine ranging from 0 to 1.2 M was tested. It has been observed that increasing betaine concentration increased the amount of the LAMP reaction products and the concentration at 0.8 M had already had the optimal amplification.

Effect of primer ratio

Since a set of LAMP primers comprise two outer and two inner primers, the

effect of ratio of outer primers and inner primers on the LAMP reaction was determined. The LAMP reaction was performed in the primer ratios ranging from 1:1 to 1:8 and a primer ratio of 1:6 gave the maximal amplification.

Effect of temperature

The LAMP reaction was performed at temperatures ranging from 53 to 65^{0}C. When the reaction temperature reached 58^{0}C, the ladder-like pattern of bands was generated, and the temperature at 60^{0}C gave the best amplification result.

Effect of reaction length

The effect of reaction lengths from 30 to 60 min. on the LAMP reaction was tested with genomic DNAs of *V. parahaemolyticus* ATCC 33846 and ATCC 33847 as templates, respectively. When the reaction performed for 45 min., the typical ladder-like pattern of bands was generated. However, in later experiments, the reaction performing for 45 min. gave inconsistent and unstable results. Therefore, we chose 60 min. as the optimal reaction length.

As indicated above, the LAMP assay condition was optimized in a 25 µl reaction mixture as follows: 8 mM $MgSO_4$, 1.0 mM dNTP, 0.8 M betaine, 1.2 µM each of FIP and BIP, 0.2µM each of F3 and B3, 1 U *Bst* DNA polymerase large fragment, 1×ThermoPol buffer and appropriate amount of template genomic DNA. The reaction was carried out at 60^{0}C for 60 min.

Specificity of LAMP assay

The specificity of the LAMP primers for the *tlh* gene was tested in *V. parahaemolyticus* and other bacterial strains with a total amount of 28. All the 14 strains of *V. parahaemolyticus* were shown to be positive, whereas, all the other bacterial strains tested in this study were negative (Table 1). These results indicated that the primers were specific for detection of *V. parahaemolyticus.*

Sensitivity of LAMP assay

The sensitivity of the LAMP assay for detection of *V. parahaemolyticus* was determined using 10-fold serial dilutions. The concentration of genomic DNA extracted from *V. parahaemolyticus* ATCC 33846 was 449 ng.µl^{-1}. Aliquots of 2 µl each dilution were used as the templates of the LAMP amplification making the concentrations of templates ranging from 90 ng. test tube^{-1} to 0.9 fg.test tube^{-1}. The detection limit of this LAMP assay for *V. parahaemolyticus* genomic DNA was around 90 fg. test tube^{-1}. The overnight cultivated *V. parahaemolyticus* ATCC 33846 was quantitated to be 2.39×10^7 cfu.mL^{-1} by direct plating. The detection limit of this LAMP assay for *V. parahaemolyticus* pure cultures was approximately 24 cfu.mL^{-1}.

Detection of V. parahaemolyticus in artificially contaminated food samples

We applied the LAMP assay to detect *V. parahaemolyticus* in artificially contaminated shrimps. The typical ladder-like pattern of bands was observed in tubes with artificially contaminated shrimps as templates, and no amplicon was observed in negative control with uncontaminated shrimps as templates.

Discussion

In this study, we developed and optimized a LAMP assay for rapid detection and identification of *V. parahaemolyticus*. LAMP amplifies DNA under isothermal conditions, which uses a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA and a DNA polymerase with high strand displacement activity. The use of four primers (recognition of six distinct sequences) in the initial steps of LAMP and two primers (recognition of four distinct sequences) during the subsequent steps ensures high specificity for the target amplification. Thus, we can judge the presence and absence of the target gene by whether the amplification performs or not. On the basis of its special amplification mechanism, the final products of LAMP are a mixture of stem-loop DNAs with various stem lengths and cauliflower-like structures with multiple loops consisting of alternate inverted repeats of target sequence in the same strand, thus, the products generate unique ladder-like pattern of bands on agarose gel (Notomi et al. 2000). Furthermore, LAMP method is able to synthesize extremely large amount of DNA, so a large amount of by-product, pyrophosphate ion, is produced, yielding white precipitate of magnesium pyrophosphate in the reaction mixture. As the precipitate can easily be observed with naked eyes, detection of LAMP reaction can be done by judging the presence of accumulated precipitate after centrifugation and visual judgment of turbidity, which may be the most direct way to judge the nucleic acid in testing specimens being amplified by LAMP method or not (Mori et al. 2001). The LAMP assay established in this study performs under isothermal condition with a single temperature step at $60^{0}C$ for approximately one hour. Moreover, it conducts amplification and detection in one-step, so the LAMP assay can be done only using a water-bath that furnishes a constant temperature of $60^{0}C$ without usage of any other expensive or specialized equipment. When compared to PCR techniques, the LAMP assay is easier to perform, less time-consuming and lower cost.

In the present study, we chose *V. parahaemolyticus* species-specific gene- *tlh* gene as the target gene. A set of LAMP primers was designed specifically to recognize the *tlh* gene. In order to confirm specificity, the assay was conducted using *V. parahaemolyticus*, bacteria from *Vibrio spp.*, and some other bacterial strains. DNA amplification was only observed in *V. parahaemolyticus*, but not other bacterial strains. This result suggests that this assay is specific for detecting *V. parahaemolyticus*.

Our LAMP assay was capable of detecting a minimum of 90 fg. test tube^{-1} for *V. parahaemolyticus* genomic DNA and 24 cfu.mL^{-1} for pure cultures. Whereas, the sensitivity of the Real-time PCR assay that recently published was 200 pg of *V. parahaemolyticus* genomic DNA and 10^4 cfu.mL^{-1} for pure cultures, and the sensitivity of conventional PCR previously reported was 2.4×10^2 cfu.mL^{-1} for pure cultures (Li et al. 2004; Ward and Bej 2006). Thus, compared to these other assays, the LAMP assay is shown to be more sensitive.

V. parahaemolyticus is an important seafood-borne pathogen throughout the world. Typically, human infections from this pathogen mainly result from the consumption of raw or undercooked seafoods. Furthermore, it is known that some components of food may inhabit DNA amplification (Hara-Kudo et al. 2005). Thus, in this study, the LAMP assay was applied to detect *V. parahaemolyticus* in artificially contaminated food samples. Artificially contaminated shrimp samples were detected *V. parahaemolyticus* positive by the LAMP assay, but not the samples that were not contaminated. This result demonstrates that the assay is capable to detect *V. parahaemolyticus* in noncultured food samples without prior isolation and biochemical speciation.

In conclusion, the LAMP assay standardized and optimized in the present study is specific and sensitive for rapid detection and identification of *V. parahaemolyticus* both in culture isolates and in food samples. When compared to traditional cultivation methods and PCR-based techniques, the LAMP assay is simpler, effective and less expensive that requires no specialized equipments. Therefore, the LAMP assay is expected to become a valuable tool for rapid detection and identification of *V. parahaemolyticus*, beneficial to both the seafood industry and consumer health.

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References

Endo, S., T. Komori, G. Ricci, A. Sano, K. Yokoyama, A. Ohori, K. Kamei, M. Franco, M. Miyaji and K. Nishimura. 2004. Detection of gp43 of Paracoccidioides brasiliensis by the loop-mediated isothermal amplification (LAMP) method. FEMS Microbiology Letters 234(1): 93-97.

Hara-Kudo, Y., M. Yoshino, T. Kojima and M. Ikedo. 2005. Loop-mediated isothermal amplification for the rapid detection of *Salmonella*. FEMS Microbiology Letters 253: 155-161.

Hirayama H., S. Kageyama, Y. Takahashi, S. Moriyasu, K. Sawai, S. Onoe, K. Watanabe, S. Kojiya, T. Notomi and A. Minamihashi. 2006. Rapid sexing of water buffalo (*Bubalus bubalis*) embryos using loop-mediated isothermal amplification. Theriogenology 66: 1249-1256.

Imai M., A. Ninomiya, H. Minekawa, T. Notomi, T. Ishizaki, P.V. Tu, N.T.K. Tien, M. Tashiro and T. Odagiri. 2006. Development of H5-RT-LAMP (loop-mediated isothermal amplification) system for rapid diagnosis of H5 avian influenza virus infection. Journal of Virological Methods 24 (44): 6679-6682.

Li Z.F., J. Nie, Y.ZH. Chen, Y.CH. Dai, J.D. Li, and C.Y. Wu. 2004. Rapid detection of *Vibrio parahaemolyticus* by PCR. Journal of Preventive Medicine of Chinese People's Liberation Army 22(6): 443-444.

Liu X.M. 2004. Studies on the techniques for the monitoring and controlling foodborne illness. Chinese Journal of Food Hygiene 16 (1): 3-9.

Mori Y., K. Nagamine, N. Tomita, T. Notomi. 2001. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochemical Biophysical Research Communications 289: 150-154.

Notomi, T., H. Okayama, H. Masubuchi, J. Yonekawa, K. Watanabe, N. Amino and T. Hase. 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Research 28(12):63.

Song, T., C. Toma, N. Nakasone, M. Iwanaga. 2005. Sensitive and rapid detection of *Shigella* and enteroinvasive *Escherichia coli* by a loop-mediated isothermal amplification method. FEMS Microbiology Letters 243: 259-263.

Su, Y.C. and C.C. Liu. 2007. *Vibrio. parahaemolyticus*: A concern of seafood safety. Food Microbiology 24(6): 549-558.

Ward, L.N. and A.K. Bej. 2006. Detection of *Vibrio parahaemolyticus* in shellfish by use of multiplexed real-time PCR with TaqMan fluorescent probes. Applied and Environment Microbiology 72(3): 2031-2042.

Yeh, H.Y., C.A. Shoemaker and P.H. Klesius. 2005. Evaluation of a loop-mediated isothermal amplification method for rapid detection of channel fish *Ictalurus punctatus* important bacterial pathogen *Edwardsiella ictaluri*. Journal of Microbiological Methods 63: 36-44.

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