Development of a toxR-based loop-mediated isothermal amplification assay for detecting *Vibrio parahaemolyticus*

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Abstract

**Background:** *Vibrio parahaemolyticus* is a leading cause of seafood-related bacterial gastroenteritis and outbreaks worldwide. Sensitive and specific detection methods are needed to better control *V. parahaemolyticus* infections. This study aimed at developing a highly specific and sensitive loop-mediated isothermal amplification (LAMP) assay for detecting *V. parahaemolyticus* in oysters. A set of five LAMP primers, two outer, two inner, and one loop were designed based on the published *V. parahaemolyticus* toxR sequence. Specificity of the assay was evaluated using a panel of 36 *V. parahaemolyticus* and 39 other strains. The assay sensitivity was determined using serial dilutions of *V. parahaemolyticus* ATCC 27969 culture ranging from 10⁸ CFU/ml to extinction. The assay was also tested in experimentally inoculated oyster samples.

**Results:** The toxR-based LAMP assay was able to specifically detect all of the 36 *V. parahaemolyticus* strains without amplification from 39 other strains. The detection limit was 47-470 cells per reaction in pure culture, up to 100-fold more sensitive than that of toxR-PCR. When applied in spiked oysters, the assay was able to detect 1.1 × 10⁵ *V. parahaemolyticus* cells per gram of oyster without enrichment, up to 100-fold more sensitive than that of toxR-PCR. Standard curves generated for detecting *V. parahaemolyticus* in both pure culture and spiked oyster samples showed good linear relationship between cell numbers and the fluorescence or turbidity signals.

**Conclusions:** The toxR-based LAMP assay developed in this study was sensitive, specific, and quantitative, holding great potential for future field detection of *V. parahaemolyticus* in raw oysters.

**Background**

The Gram-negative, halophilic marine bacterium *Vibrio parahaemolyticus* has emerged as a major cause of seafood-associated outbreaks throughout the world and become a significant concern of seafood safety [1-3]. Shellfish, particularly oysters, has been frequently implicated in *V. parahaemolyticus* infections [4,5]. Typically within 24 h after eating contaminated seafood, *V. parahaemolyticus* causes acute, self-limiting gastroenteritis characterized by diarrhea, abdominal cramps, nausea, vomiting, fever, and chills, which lasts for 1-3 days [6]. Two hemolysins, the thermostable direct hemolysin (TDH) and the TDH-related hemolysin (TRH) are well-characterized virulence factors for pathogenic *V. parahaemolyticus* strains [7]. However, the majority of *V. parahaemolyticus* strains in the environment and seafood samples lack these two hemolysin genes [8-10], thus the number of total *V. parahaemolyticus* has been used as an indicator for preventing *V. parahaemolyticus* infections from seafood consumption [11,12].

Traditional culture-based methods for isolating and enumerating *V. parahaemolyticus* from seafood samples involve the most probable number (MPN) technique [13]. Although widely used, such methods are labor-intensive and time-consuming (4-7 days). Molecular-based methods such as DNA probe hybridization and PCR assays have been developed for *V. parahaemolyticus* and yielded rapid and specific results [14-18]. However, the probe hybridization procedure and the gel electrophoresis technique used to analyze PCR amplions are tedious and time-consuming. Recently, several real-time PCR assays have been developed for the detection of *V. parahaemolyticus* with increased speed and...
sensitivity [12,19-21]. Nonetheless, these assays require a dedicated real-time PCR machine, which is rather expensive and not yet widely available.

Loop-mediated isothermal amplification (LAMP), a novel DNA amplification technique invented in 2000 [22], has since been applied in detecting many bacterial and viral agents [23-26]. Because the LAMP assay was carried out under isothermal conditions, a simple heater that maintains a constant temperature (60-65°C) is sufficient. LAMP assays were reported to be highly specific, sensitive, rapid, and cost-effective [23-26]. Very recently, LAMP was adopted to detect sensitive, rapid, and cost-effective [23-26]. Very recently, LAMP was adopted to detect V. parahaemolyticus and yielded promising results [11]. However, in this LAMP assay, primers were designed to target the V. parahaemolyticus thermolabile hemolysin gene (tlh), and an increasing number of hemolysin gene sequences with various levels of identity to this gene have been described in other vibrios such as Vibrio harveyi and Vibrio campbellii [27,28]. A search of the GenBank also revealed significant homologies among these hemolysin genes http://www.ncbi.nlm.nih.gov/BLAST. Additionally, Croci et al. [29] evaluated several PCR assays for the identification of V. parahaemolyticus by targeting different genes. Among 48 V. parahaemolyticus and 115 other Vibrio spp. strains examined, the two tlh-based PCR protocols [13,14] obtained 100% inclusivity but had 50% and 91% exclusivity, respectively. In contrast, a toxR-based PCR assay [18] simultaneously evaluated in the same study achieved 100% for both inclusivity and exclusivity [29].

The toxR gene was initially described in V. cholerae as the regulatory gene for the cholera toxin and other virulence determinants [30], and was subsequently found in V. parahaemolyticus [31]. Although present in many Vibrio spp., a membrane “tether” region within the coding sequence of toxR possesses significant heterogeneity and could be used to distinguish various Vibrio species [32]. The objective of this study was to develop a highly specific and sensitive toxR-based LAMP assay for the detection of V. parahaemolyticus in raw oyster samples.

Results

Specificity of the LAMP assay

The V. parahaemolyticus toxR-based LAMP assay run on two platforms by using either a real-time PCR machine or a real-time turbidimeter successfully detected 36 V. parahaemolyticus strains while showing negative results for 39 non-V. parahaemolyticus strains (Table 1), indicating that the toxR-based LAMP assay was highly specific. On the real-time PCR platform, mean cycle threshold (Ct; cycles when fluorescence signals reach 30 units) values for the 36 V. parahaemolyticus clinical and environmental strains ranged between 13.58 and 23.95 min, with an average of 17.54 ± 2.27 min. The melting temperatures (Mt) for these strains consistently fell between 81.25 and 82.55°C, with an average Mt of 81.97 ± 0.25°C. For the 39 non-V. parahaemolyticus strains, no Ct value was obtained, with melting curve analysis showing no peaks, suggesting no amplification occurred.

On the real-time turbidimeter platform, time threshold (Tt; time when turbidity values reach 0.1) values for the 36 V. parahaemolyticus clinical and environmental strains ranged from 28.3 to 33.5 min with an average of 31.13 ± 1.67 min. For the 39 non-V. parahaemolyticus strains, no Tt value was obtained, indicating negative results for V. parahaemolyticus toxR-based LAMP assay. Similarly, no false positive or false negative results for the 75 bacterial strains were observed by PCR using two primer sets, F3/B3 and toxR-PCR primers (Table 2), indicating good specificity.

Sensitivity of the LAMP assay

Figure 1 presents sensitivity of the toxR-based LAMP assay when testing 10-fold serial dilutions of V. parahaemolyticus ATCC 27969 DNA templates. A representative optic graph and corresponding melting curve analysis for the real-time PCR platform and a representative turbidity graph for the real-time turbidimeter platform are shown in Figure 1A-1C, respectively. On the real-time PCR platform, for templates ranging in concentration from 4.7 × 10^5 to 4.7 × 10^1 CFU per reaction tube, the average Ct values of six repeats ranged from 17.35 to 40.72 min, with melting temperatures consistently falling at around 83°C. No amplification was obtained for the 4.7 CFU and 4.7 × 10^1 CFU templates. Therefore, the detection limit of the toxR-based LAMP assay run in a real-time PCR machine was approximately 47 CFU per reaction. In the real-time turbidimeter platform, the average Tt values fell between 34.43 and 49.07 min for templates ranging from 4.7 × 10^5 to 4.7 × 10^2 CFU per reaction tube. In two out of six repeats, amplification of the 4.7 × 10^1 CFU template occurred (Figure 1C). Therefore, the lower limit of detection for turbidity-based real-time LAMP assay was 47-470 CFU per reaction.

The two PCR assays used to test the same set of V. parahaemolyticus ATCC 27969 templates by using F3/B3 and toxR-PCR primers had the same level of sensitivity, approximately 4.7 × 10^3 CFU per reaction tube (data not shown), i.e., up to 100-fold less sensitive than the toxR-based LAMP assay.

Quantitative capability of LAMP for detecting V. parahaemolyticus in pure culture

Figure 2 shows the standard curves generated when detecting V. parahaemolyticus ATCC 27969 in pure culture based on six independent repeats in both real-time
### Table 1 Bacterial strains used in this study

| Strain group               | Strain ID and serotype | Source and reference                      |
|----------------------------|------------------------|------------------------------------------|
| *V. parahaemolyticus*      | ATCC 17802; O1:K1     | Shirasu food poisoning, Japan            |
| *(n = 36)*                 | ATCC 27969            | Blue crab, Maryland                      |
|                            | ATCC 33847            | Gastroenteritis, Maryland                |
|                            | ATCC 49529; O4:K12    | Feces, California                        |
|                            | CT-6636; O3:K6        | Clinical, Connecticut                    |
|                            | M350A, O5             | Oyster, Washington                       |
|                            | NY477, O4K8           | Oyster, New York                         |
|                            | TX-2103; O3:K6        | Clinical, Texas                          |
|                            | 8332924, O1:K56       | Oyster, Gulf of Mexico                   |
|                            | 83A0875               | Clinical, feces                          |
|                            | 83A09148              | Clinical, feces                          |
|                            | 83A09756; O4:K12      | Clinical, feces                          |
|                            | 84A01516; O4:K12      | Clinical, feces                          |
|                            | 84A04226              | Clinical, feces                          |
|                            | 916i, 916e, 541-0-44c, V68, V69, V154, V155, V166 | Oyster, Gulf, Louisiana [10] |
|                            | VS, V15, V16, V32, V38, V39, V50, V66, V150, V426, V427, V428, V429, V430 | Oyster, Retail, Louisiana [10] |
| *V. vulnificus*            | ATCC 27562            | Blood, Florida                           |
| *(n = 18)*                 | ATCC 29306            | Corneal ulcer, Virginia                  |
|                            | ATCC 33815            | Leg ulcer, Wisconsin                     |
|                            | ATCC 33816            | Blood, Alaska                            |
|                            | C7184                 | Thumb drainage, Texas [39]               |
|                            | 1003                  | Wound, Louisiana [40]                    |
|                            | 1006                  | Blood, Louisiana [40]                    |
|                            | WR1                   | Sea water, Washington                    |
|                            | S15-4C2               | Oyster, California                       |
|                            | S41-0-84c             | Gulf oyster, Louisiana [10]              |
|                            | V373, V416, V578, V598 | Retail oyster, Louisiana [10]            |
|                            | 132A1, 132T5, 212B6, 342E3 | Gulf oyster, Louisiana[6] |
| Other *Vibrio* spp. *(n = 10)* | ATCC 17749           | Spoiled horse mackerel, Japan            |
|                            | ATCC 33787            | Seawater, Hawaii                         |
| *Vibrio alginiticus*       | ATCC 14035; O:1       | United Kingdom                           |
| *Vibrio cholerae*          | ATCC 35912            | Blood/cerebrospinal fluid, Ohio          |
| *Vibrio cincinnatiensis*   | ATCC 33809            | Human feces, Bangladesh                  |
| *Vibrio harveyi*           | ATCC 14126            | Dead amphipod, Massachusetts             |
|                            | ATCC 35084            | Brown shark, Maryland                    |
| *Vibrio mimicus*           | ATCC 33653            | Human ear, North Carolina                |
|                            | ATCC 33655            | Feces, Tennessee                         |
| *Vibrio natriegens*        | ATCC 14048            | Salt marsh mud, Georgia                  |
| Non-*Vibrio* spp. *(n = 11)* | ATCC 81-176           | Human                                    |
| *Campylobacter jejuni*     | ATCC 13048            | Sputum, South Carolina                   |
| *Enterobacter aerogenes*   | ATCC 29212            | Urine                                    |
| *Escherichia coli*         | ATCC 25922            | Human                                    |
| *Listeria monocytogenes*   | ATCC 13932; 4b        | Spinal fluid, Germany                    |
| *Pseudomonas aeruginosa*   | ATCC 27853            | Human blood                              |
| *Salmonella enterica*      | LT2; Typhimurium      | Unknown                                  |
| *Shigella flexneri*        | ATCC 12022; 2b        | Unknown                                  |
| *Shigella sonnei*          | ATCC 25931            | Human feces, Panama                      |
| *Staphylococcus aureus*    | ATCC 29213            | Wound                                    |
| *Streptococcus pneumoniae* | ATCC 49619; type 59   | Sputum, Arizona                          |

*a* ATCC, American Type Culture Collection, Manassas, VA. *b* Isolated from three Louisiana coastal locations (designated as 132, 212, and 342) between 2006-2007.
PCR machine (Figure 2A) and a real-time turbidimeter (Figure 2B). On the real-time PCR platform, the correlation coefficient ($r^2$) was calculated to be 0.95. When run in the real-time turbidimeter platform, the toxR-based LAMP assay had an $r^2$ value of 0.94.

Detection of *V. parahaemolyticus* cells in spiked oysters
The sensitivity of detecting *V. parahaemolyticus* ATCC 27969 cells in spiked oyster samples is shown in Table 3. In three independent spiking experiments, the toxR-based LAMP assay using the two platforms consistently detected down to 200 *V. parahaemolyticus* cells (i.e., $1.1 \times 10^5$ CFU/g) in spiked oyster samples without enrichment. However, for the two PCR assays using F3/B3 and toxR-PCR primers, the lowest detection limit achieved was $1.1 \times 10^6$ CFU/g and $1.1 \times 10^7$ CFU/g, which were up to 100-fold less sensitive than that of the toxR-based LAMP assay. Standard curves (Figure 3) generated for

| Primer name | Sequence (5'-3') | Positiona | Amplicon size (bp) | Reference |
|-------------|-----------------|-----------|-------------------|-----------|
| F3          | TTGGATTCCACGGGTAT | 528-545   | Ladder-like bands for LAMP; 183 bp for F3/B3 PCR | This study |
| B3          | CGTTCAATGCACGTCTA | 693-710   |                   |           |
| FIP         | TGGATTCGCCAGGGCTTAA | 587-608  | (F1c)             |           |
|             | TTATTTTGGCACATTATTACCG | 547-570  | (F2)              |           |
| BIP         | GGTCCGTCAATGCTGTTACGTCAT | 609-631 | (B1c)             |           |
|             | TAGAAGGCAAACCCAGTGTGTT | 673-691 | (B2)              |           |
| Loop        | AGAACGTACAGATGACC | 632-653   |                   |           |
| toxR-F      | GTCTCTGAGCAATCGTTG | 453-472   | 367b              | [18]      |
| toxR-R      | ATACGAGTGGCTGCATG | 799-819   |                   |           |

a The positions are numbered based on the coding sequence of *V. parahaemolyticus* strain AQ3815 toxR gene [GenBank: L11929].

b Differences in primer positions and amplicon size were noted from those originally published after reanalysis of the sequences.

Figure 1 Sensitivity of the LAMP assay when detecting *Vibrio parahaemolyticus* ATCC 27969 in pure culture. (A) A representative optical graph generated using the real-time PCR machine; (B) Corresponding melting curve analysis for samples in (A); (C) A representative turbidity graph generated using the real-time turbidimeter. Samples 1-7 correspond to serial 10-fold dilutions of *V. parahaemolyticus* ATCC 27969 cells ranging from $4.7 \times 10^5$ to $4.7 \times 10^{-1}$ CFU/reaction; sample 8 is water.
Figure 2 Standard curves generated when detecting *Vibrio parahaemolyticus* ATCC 27969 in pure culture. (A) Based on six independent repeats in a real-time PCR machine; (B) Based on six independent repeats in a real-time turbidimeter.

Table 3 Comparison of quantitatively detecting *Vibrio parahaemolyticus* ATCC 27969 in spiked oysters by using the toxR-based LAMP assay in two platforms and PCR

| Rep. | Levels of spiking (CFU/g) | Amount of cellsb (CFU/rxn) | LAMP | PCR |
|------|-------------------------|---------------------------|------|-----|
|      |                         |                           | Fluorescence-based | Turbidity-based | F3/B3 | toxR |
|      |                         |                           | Ct (min) | Mt (°C) | Tt (min) |
| 1    | 1.1 × 10⁶              | 2.0 × 10⁷                 | 35.57 ± 1.73 | 82.65 ± 0.09 | 47.25 ± 0.35 | - |
|      | 1.1 × 10⁵              | 2.0 × 10⁶                 | 33.26 ± 1.06 | 82.50 ± 0.26 | 44.8 ± 0.7 | - |
|      | 1.1 × 10⁴              | 2.0 × 10⁵                 | 31.77 ± 2.23 | 82.50 ± 0.18 | 47.7 ± 1.27 | - |
|      | 1.1 × 10³              | 3.1 × 10³                 | 29.08 ± 0.45 | 82.60 ± 0.34 | 40.4 ± 4.67 | - |
| 2    | 1.7 × 10⁶              | 3.1 × 10⁶                 | 31.77 ± 2.23 | 82.50 ± 0.18 | 47.7 ± 1.27 | - |
|      | 1.7 × 10⁵              | 3.1 × 10⁵                 | 29.08 ± 0.45 | 82.60 ± 0.34 | 40.4 ± 4.67 | - |
|      | 1.7 × 10⁴              | 3.1 × 10⁴                 | 23.68 ± 0.16 | 82.25 ± 0.10 | 33.25 ± 0.35 | + |
|      | 1.7 × 10³              | 3.1 × 10³                 | 21.78 ± 0.59 | 82.41 ± 0.11 | 29.4 ± 0.85 | + |
|      | 1.7 × 10²              | 3.1 × 10²                 | 1.0 ± 10⁰   | 82.16 ± 0.05 | 31.2 ± 2.97 | + |
|      | 1.7 × 10¹              | 3.1 × 10¹                 | 32.6 ± 0.26  | 82.48 ± 0.01 | 31.25 ± 4.02 | + |

| Bolded are detection limits by each assay. |
| b For each independently prepared template, two times of LAMP reactions were performed and the data presented are means ± standard deviations for the 2 LAMP repeats. |
| c CFU/reaction was calculated by using CFU/g × 0.09 g/ml × 10 × 2 × 10⁻³, i.e., CFU/g × 1.8 × 10⁻³. |
the quantitative detection of *V. parahaemolyticus* cells in spiked oyster samples had an $r^2$ value of 0.99 for both real-time LAMP platforms.

**Discussion**

In this study, we designed a set of five LAMP primers to specifically target the *V. parahaemolyticus* toxR gene, a gene previously shown to possess better specificity for *V. parahaemolyticus* detection by PCR than other target genes, such as *tlh* and *gyrB* [29]. We also developed real-time LAMP assays using two platforms - a real-time PCR machine and a real-time turbidimeter to quantitatively detect *V. parahaemolyticus* in pure culture and spiked oyster samples. This is the first report demonstrating the efficacy of a toxR-based LAMP assay for detecting *V. parahaemolyticus* in oysters.

The LAMP primers were selected from regions of the *V. parahaemolyticus* toxR gene coding sequence that are highly specific to *V. parahaemolyticus* [18,32]. The five primers (F3, B3, FIP, BIP, and loop) targeted seven regions of *V. parahaemolyticus* toxR (Table 2), providing additional levels of specificity compared to PCR primers (targeting two regions). Among a total of 36 *V. parahaemolyticus* and 39 non-*V. parahaemolyticus* strains tested, the toxR-based LAMP assay run on both real-time platforms obtained 100% inclusivity and 100% exclusivity. This level of specificity was the same as that of two toxR-based PCR assays evaluated simultaneously in this study and that of a *tlh*-based LAMP assay developed by Yamazaki et al. [11]. Future pairwise comparison of the two LAMP assays (toxR-based and *tlh*-based) using an extensive collection of *Vibrio* strains as done previously for PCR [29] would be desired to further evaluate the performance of the two LAMP assays on both inclusivity and exclusivity.

When comparing the sensitivity of LAMP with PCR, the toxR-LAMP assays were able to detect 47–470 *V. parahaemolyticus* cells per reaction tube, in contrast to $4.7 \times 10^3$ cells for toxR-PCR. Similarly, the *tlh*-based LAMP assay for *V. parahaemolyticus* was reported to be 10-fold more sensitive than PCR, with a detection limit of 2 CFU per reaction for LAMP [11]. In a recent report on the detection of pathogenic *V. parahaemolyticus* by targeting the *tdh* gene, both LAMP and PCR were capable of detecting less than 1 CFU of TDH-producing *V. parahaemolyticus* in a reaction tube, although for different serotypes tested, slight difference in terms of sensitivity was observed [33]. Additionally, several studies on the detection of other *Vibrio* spp. also found LAMP to be 10-fold more sensitive than PCR [23,34,35].

Running the toxR-LAMP assay in a real-time PCR machine consistently achieved a lower limit of detection of 47 cells per reaction, whereas in a real-time turbidimeter, a detection limit of 47 cells was only occasionally achieved (2 out of 6 attempts). In addition, the average time to positive results as indicated by $Ct$ (17.54 min)
for the real-time PCR platform was markedly shorter than that of the real-time turbidimeter platform as indicated by $Tt$ (31.13 min), suggesting that the real-time LAMP assay based on fluorescence was faster and slightly more sensitive than that based on turbidity. This finding agrees with a previous study which reported that a fluorescent intercalation dye (YO-PRO-1)-based real-time LAMP was 10-fold more sensitive and faster than a turbidimetry real-time LAMP [36]. However, in that report, the fluorescence-based LAMP assay was found to generate anomalous and irreproducible results in low-concentration templates (less than $1 \times 10^3$ copies), which could be due to the effect of the intercalating dye on DNA amplification efficiency [36].

In this study, we chose SYTO-9 as the intercalating dye for the real-time PCR platform instead of the commonly used real-time PCR dye SYBR Green I. Based on a previous study [37] comparing the use of these two dyes in real-time PCR, SYTO-9 was found to generate highly reproducible DNA melting curves over a broader range of dye concentrations than SYBR Green I and was far less inhibitory. We also evaluated the use of EvaGreen (Biotium, Hayward, CA) as the intercalating dye on the real-time PCR platform for LAMP, but found it to be inhibitory for LAMP amplifications (data not shown).

The strong linear correlation ($r^2 = 0.94-0.99$) between the number of *V. parahaemolyticus* cells in the LAMP reaction and the associated $Ct$ or $Tt$ values over a dynamic range of template concentrations ($10^1$ to $10^6$ cells) illustrates the quantitative capability of the *toxR*-based real-time LAMP assays when detecting this organism in both pure culture and spiked oysters. Very few reports have examined the quantitative ability of LAMP. One study monitoring ammonia-oxidizing bacteria using LAMP also reported it to possess good quantitative capability in both pure culture and spiked oysters. Very few studies have examined the quantitative ability of LAMP. One study monitoring ammonia-oxidizing bacteria using LAMP also reported it to possess good quantitative capability in both pure culture and spiked oysters. Very few studies have examined the quantitative ability of LAMP.

When testing spiked oyster samples, we observed the time to positive samples ($Ct$ for the real-time PCR platform and $Tt$ for the real-time turbidimeter) was delayed several minutes compared to pure culture samples and the detection limit was higher (200 *V. parahaemolyticus* cells in oyster samples vs. 47 cells in pure culture). Nonetheless, no extensive sample preparation other than homogenization and two simple centrifugation steps was required. This significantly reduced the total assay time. Combined with less than 1 h for the real-time LAMP assay, the complete LAMP detection system was markedly faster than either PCR or conventional methods.

**Conclusions**

The *toxR*-based real-time LAMP assay developed in this study was a highly specific, sensitive, and rapid method for the detection of *V. parahaemolyticus* in oysters. Future testing with natural or commercial oyster samples is desired to further evaluate the efficacy of the assay in detecting *V. parahaemolyticus* in oysters in a field setting.

**Methods**

**Bacterial strains and DNA templates preparation**

Strains used in this study (Table 1) were maintained in Luria-Bertani broth (BD Diagnostic Systems, Sparks, MD) containing 30% glycerol at -80°C. *V. parahaemolyticus* ATCC 27969, originally isolated from blue crab hemolymph was used for sensitivity testing. Additional 35 *V. parahaemolyticus* clinical and environmental strains and 39 non-*V. parahaemolyticus* strains were used to evaluate assay specificity. All *Vibrio* strains were routinely cultured using trypticase soy agar or broth (TSA or TSB; BD Diagnostic Systems) supplemented with 2% NaCl at 35°C overnight. Non-*Vibrio* strains were grown on Luria-Bertani agar or blood agar (BD Diagnostic Systems).

To prepare DNA template, a single bacterial colony grown on appropriate agar plates was suspended in 500 μl of TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA; Sigma-Aldrich, St. Louis, MO) and heated at 95°C for 10 min in a dry heating block. The crude cell lysate was centrifuged at 12,000 g for 2 min and the supernatant was stored at -20°C until use.

**LAMP primers and reaction conditions**

The *V. parahaemolyticus toxR* gene [GenBank: L11929] was used as the target for LAMP primer design. Five primers, two outer (F3 and B3), two inner (FIP and BIP), and one loop (Loop) which recognized seven distinct regions of the target sequence were designed using the PrimerExplorer software version 4 (Fujitsu Limited, Japan; http://primerexplorer.jp/e. Oligonucleotide sequences and locations of the primers are shown in
Table 2. The primers were synthesized by Invitrogen (Carlsbad, CA).

The LAMP reaction mix in a 25 μl total volume consisted of the following: 1 × Thermo buffer, 6 mM of MgSO4, 0.8 M of betaine (Sigma-Aldrich), 1.4 mM of deoxynucleotide triphosphate (dNTP), 0.2 μM of each outer primer (F3 and B3), 1.6 μM of each inner primer (FIP and BIP), 0.8 μM of the loop primer, 8 U of Bst DNA polymerase (New England Biolabs, Ipswich, MA), and 2 μl of DNA template. Additionally, 0.4 μM of SYTO-9 green fluorescent dye (Invitrogen) was added when the LAMP reaction was carried out in a real-time PCR machine as described below.

Two platforms were used to run the LAMP reactions. On the first platform, a real-time PCR machine (SmartCycler II System; Cepheid, Sunnyvale, CA) was used and the SYTO-9 green fluorescent dye was added. The assay was conducted at 63°C for 1 h. Fluorescence readings were acquired every 60 s using the FAM channel (excitation at 450-495 nm and detection at 510-527 nm), followed by melting curve analysis from 63°C to 96°C with 0.2°C increment per second. The fluorescence threshold unit was set to be 30. On the second platform, the LAMP reaction was carried out in a Loopamp real-time turbidimeter (LA-320C; Teramecs, Kyoto, Japan) at 63°C for 1 h and terminated at 80°C for 5 min. Turbidity readings at 650 nm were obtained real-time and a turbidity threshold value of 0.1 was used. A negative control was included for each LAMP run.

PCR
As a comparison, two sets of PCR reactions were performed, one using LAMP outer primers (F3 and B3) and the other one using the toxR-PCR primers (Table 2) published previously [18]. Each PCR mix in a 25 μl total volume contained 1 × PCR buffer, 0.2 μM of each dNTP, 1.5 mM of MgCl2, 0.5 μM of each forward and reverse primer, 0.625 U of GoTaq Hot Start Polymerase (Promega, Madison, WI), and 2 μl of DNA template. The PCR reactions were conducted using initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 60°C (50°C for F3/B3 primers) for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 7 min in a Bio-Rad C1000 Thermal Cycler (Hercules, CA). Aliquots (10 μl) of PCR products were analyzed by electrophoresis on 1.5% agarose gel containing ethidium bromide, and visualized under UV light. Gel images were documented by a Gel Doc XR system (Bio-Rad).

LAMP specificity and sensitivity
Seventy-five bacterial strains (Table 1) were used to determine the LAMP specificity. DNA templates were made from fresh overnight bacterial cultures and aliquots (2 μl) were subjected to both LAMP and PCR amplifications. Specificity tests were repeated twice.

To determine LAMP sensitivity, serial 10-fold dilutions (ca. 10⁸ CFU/ml to extinction) of a mid-log phase V. parahaemolyticus ATCC 27969 culture grown in TSB were prepared in phosphate buffered saline (PBS; BD Diagnostic Systems) and quantified using the standard plating method. DNA templates were prepared from each dilution by the boiling method described above and aliquots (2 μl) were subjected to both LAMP and PCR amplifications. Sensitivity tests were repeated six times and the lower limits of detection (CFU/reaction) were reported. Standard curves were generated by plotting Ct (cycle threshold; for the real-time PCR platform) or Tt (time threshold; for the real-time turbidimeter platform) values against log CFU/reaction and the linear regression was calculated using the Microsoft Excel Software (Seattle, WA).

LAMP testing in experimentally inoculated oyster samples
Oyster samples were obtained from local seafood restaurants and determined to be V. parahaemolyticus-negative as described previously [10]. Oyster samples were processed following a previous study with slight modifications [11]. Briefly, 25 g of oyster sample was mixed with 225 ml of alkaline peptone water (APW; BD Diagnostic Systems) and homogenized in a food stomacher (Model 400; Tekmar Company, Cincinnati, OH) for 90 s to generate 1:10 oyster in APW homogenate. Serial 10-fold dilutions of a mid-log phase V. parahaemolyticus ATCC 27969 culture were prepared in PBS as described above. Aliquots (100 μl) of each dilution were inoculated into 900 μl of 1:10 oyster in APW homogenate. The spiked oyster samples were mixed well and centrifuged at 900 g for 1 min to remove oyster tissues. The supernatants were transferred to a fresh tube and centrifuged at 10,000 g for 5 min to pellet bacterial cells. After removing the supernatants, pellets were resuspended in 100 μl of TE and boiled for templates as described above. Aliquots (2 μl) of the supernatant were used for both LAMP and PCR amplifications. The spiked oyster sensitivity tests were repeated three times and the lower limits of detection (CFU/g) were reported. Standard curves were generated similarly as in pure culture sensitivity testing.

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Authors’ contributions
SC carried out the LAMP and PCR assays, conducted data analysis, and drafted the manuscript; SC and BG conceived of the study and participated in its design. BG coordinated the study and helped to finalize the manuscript. Both authors read and approved the final manuscript.
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48(2)

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