A Real-Time Loop-Mediated Isothermal Amplification Assay for Detecting Virulent-Type *Vibrio vulnificus* Strains in Oysters

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Introduction

Vibrio vulnificus is a Gram-negative, halophilic bacterium that inhabits warm coastal and estuarine waters throughout the world (Oliver 2006). Rapid and sensitive detection assays are needed to facilitate better control of potential *V. vulnificus* infections from oyster consumption.

Epidemiological data suggested that only a small percentage of *V. vulnificus* strains in oysters are virulent (Jackson, Murphree et al. 1997); however, unique virulence markers have not been identified. Recently, a virulence-correlated gene (*vcg*) in *V. vulnificus* has been explored to differentiate virulent- (i.e. clinical-; *vcgC*) from non-virulent- (i.e. environmental-; *vcgE*) type *V. vulnificus* strains (Warner and Oliver 2008).

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification assay developed in 2000 that utilizes four to six primers to specifically recognize six to eight regions of the target DNA sequence and amplify millions of DNA copies under isothermal conditions (60-65^{o}C) within an hour (Notomi, Okayama et al. 2000). Additionally, LAMP can be quantitative (i.e., real-time LAMP) by measuring the formation of a by-product (magnesium pyrophosphate) during LAMP amplification, which correlates with the amount of amplified DNA and could be monitored by a real-time turbidimeter (Mori, Nagamine et al. 2001). The present study aimed to develop a real-time-LAMP assay suitable for the quantitative detection of virulent-type *V. vulnificus* in raw oysters by targeting *vcgC*.

Materials and Methods

The *V. vulnificus vcgC* (GenBank accession number AY626575) was selected and optimized as the target for LAMP primer design. *V. vulnificus* clinical strain ATCC 33815 (*vcgC* +) was used for the sensitivity testing in pure culture and spiked oysters (without and with enrichment). Another 33 *vcgC* + and 50 *vcgC* - (i.e., *vcgE* type) *V. vulnificus*, as well as 30 other *Vibrio* and 12 non-*Vibrio* strains were used to evaluate *vcgC* LAMP assay specificity.

The real-time LAMP was carried out at 65^{o}C for 1 h and terminated at 80^{o}C for 5 min in a real-time turbidimeter. As a comparison, real-time PCR and PCR using the *vcgC* LAMP outer primers (F3/B3) gene were performed.

The real-time LAMP was evaluated in both spiked and natural oyster samples.

Results and Discussion

This is the first report examining the quantitative capability of real-time LAMP for the detection of virulent-type *V. vulnificus* in oysters by targeting *vcgC*.

Real-time LAMP optimization. The optimized LAMP assay reagent mix and reaction condition for *vcgC* were: 6 mM $MgCl_2$, 1.2 mM dNTP, 10 U *Bst* DNA

polymerase, 2.0 μM each inner primer, 0.05 μM each outer primer, and 1.0 μM each loop primer at 65°C for 40 min.

Specificity of the real-time LAMP assay. Among 125 bacterial strains used to determine LAMP specificity, no false positive or false negative results were observed. The *Tt* values for the 33 *vcgC*-type *V. vulnificus* ranged from 16.1 to 22.3 min with an average of 18.17 ± 1.45 min. For the other 92 strains, no *Tt* value was obtained, indicating high specificity.

Sensitivity and quantitative capability of the real-time LAMP assay. Fig. 1 presents sensitivity of *vcgC* real-time LAMP, real-time PCR, and PCR amplification when testing 10-fold serial dilutions of *V. vulnificus* ATCC 33815 DNA templates. For templates ranging from 5.4×10^4 to 5.4 CFU/reaction, based on five repeats, the average *Tt* values fell between 17.5 and 31.0 min. In two out of five repeats, amplification of the 0.5 CFU template occurred. Therefore, the limit of detection for the real-time LAMP assay was 0.5-5.4 CFU/reaction. Based on the standard curve (Fig. 1B), the real-time LAMP assay had an r^2 value of 0.97.

When testing the same set of *V. vulnificus* ATCC 33815 templates by real-time PCR using *vcgC* F3/B3, real-time PCR and PCR both could detect 5.4×10^2 CFU/reaction (Fig. 1C and 1D), which were up to 1,000-fold less sensitive than real-time LAMP assay. We previously reported a *vvhA*-based LAMP sensitivity of 20 CFU/reaction and a PCR sensitivity of 202 CFU/reaction (Han and Ge 2008) and further a real-time LAMP that could detect 1-10 CFU/reaction and a PCR comparison of 100 CFU/reaction (Han and Ge 2010), which are comparable to findings of this study.

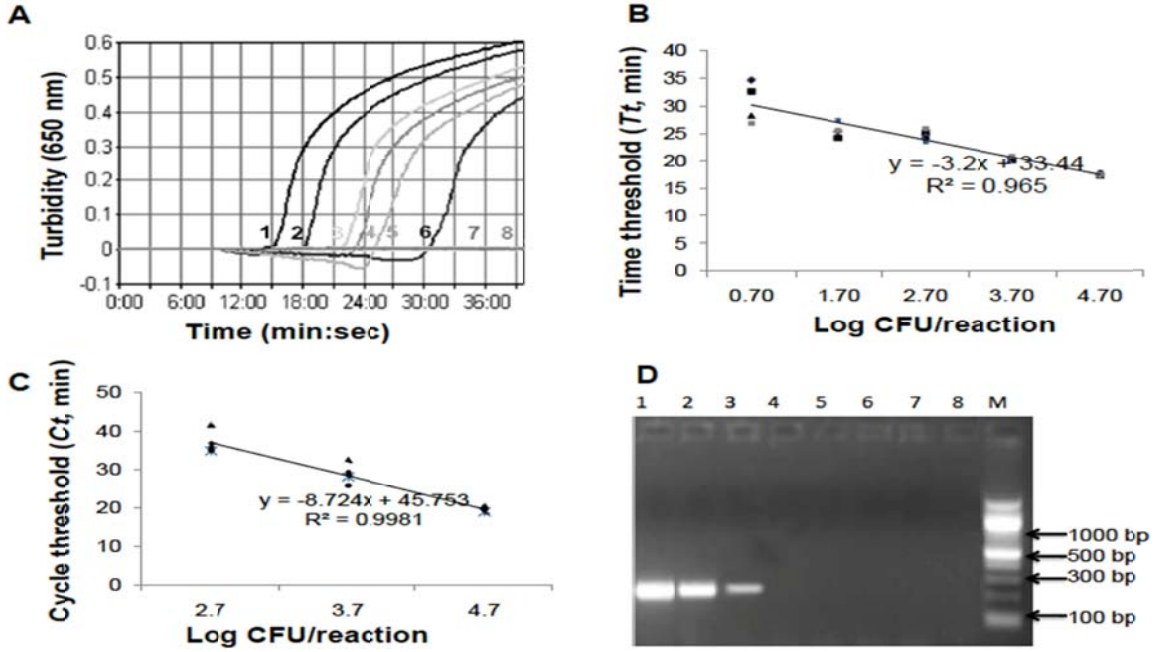

Fig. 1. Sensitivity of real-time LAMP, real-time PCR, and PCR when testing serial-diluted *Vibrio vulnificus* ATCC 33815 DNA templates. (A) An amplification graph of real-time LAMP in turbidimeter. (B) A standard curve of real-time LAMP. (C) A standard curve of real-time PCR. (D) PCR using F3/B3 primer (226 bp). Samples (1-7) correspond to 10-fold serial dilutions of *V. vulnificus* ATCC 33815 cells ranging from 5.4×10^4 to 0.05 CFU/reaction; sample 8 is water. M is molecular DNA marker.

Real-time LAMP in spiked oysters. In three independent spiking experiments, the limit of detection by real-time LAMP assay was 4.52 CFU/reaction (i.e., 2.5×10^3 CFU/g) of *V. vulnificus* ATCC 33815 in spiked oyster samples without enrichment. Standard curves generated for the quantitative detection of *V. vulnificus* cells in spiked oyster samples had an r^2 value of 0.99. After 6h enrichment, the real-time LAMP could detect 1.2 CFU/g based on three independent experiments.

Real-time LAMP in natural oysters. Among fourteen natural oysters tested, the real-time LAMP results showed negative results for direct testing, but positive after 6 h of enrichment with *Tt* values ranging from 28.3 to 59.2 min.

Conclusions

The real-time LAMP assays targeting *vcgC* developed in this study was a highly specific, sensitive, rapid, and quantitative method for the detection of virulent-type *V. vulnificus* in oysters. The detection limit of the real-time LAMP assay was 0.5-5 CFU in pure cultures and 2.5×10^3 CFU/g of *V. vulnificus* without enrichment. After 6h enrichment, the real-time LAMP assay was able to detect 1.2 CFU/g of *V. vulnificus*. Standard curves generated in both pure culture and spiked oyster samples showed good linear relationship between virulent-type *V. vulnificus* cell counts and the turbidity signals. This assay may facilitate regulatory and oyster industry personnel to better control potential *V. vulnificus* risks associated with oyster consumption.

Acknowledgements

This study was supported in part by a research grant (R/PMO-20) from the Louisiana Sea Grant College Program, with funds from the National Oceanic and Atmospheric Administration Office of Sea Grant, U.S. Department of Commerce.

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