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Virulence of thermolable haemolysi *tlh*, gastroenteritis related pathogenicity *tdh* and *trh* of the pathogens *Vibrio Parahemolyticus* in Viable but Non-Culturable (VBNC) state

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ABSTRACT

In the Viable but Non-Culturable (VBNC) state, microorganisms may survive under severe external environment. In this study, the specificity and sensitivity of PMA-LAMP assay on the detection of *Vibrio Parahemolyticus* (*V. parahemolyticus*) has been developed and evaluated, with further application on a number of food-borne *V. parahemolyticus* strains. Six primers were designed for recognizing 8 distinct targeting on *tlh*, *tdh* and *trh* gene. Through specific penetration through the damaged cell membrane of dead cells and intercalating into DNA, PMA could prevent DNA amplification of dead bacteria from LAMP, which enabled the differentiation of bacteria between VBNC state and dead state. The established PMA-LAMP showed significant advantage in rapidity, sensitivity and specificity, compared with regular PCR assay. The applicability had also been verified, demonstrating the PMA-LAMP was capable of detection on *V. parahaemolyticus*.

1. Introduction

The latest surveillance of food-borne disease shows that the food-borne pathogen accounted for more than 80% diseases in food industry. The resistance of food-borne pathogens in adverse environment harms food contamination and further microbial control. The Viable but Non-Culturable (VBNC) state is a unique survival strategy for the non-spore-forming bacteria against adverse environment, which differs from the transition state [28]. Bacteria in VBNC state cannot grow on the heterotrophic plate, but still possess metabolic activity, pathogenicity and toxicity [1,2]. Bacteria in VBNC state is capable of recovering and restoring for cultivation [10] and pathogenicity when in suitable condition [3–7]. However, investigations [2,9] has indicated that the VBNC state of bacteria still has potential virulence.

As a major concern in food safety, *Vibrio Parahemolyticus* (*V. parahaemolyticus*) is a halophilic, Gram-negative bacterium, responsible for 50–70% of all diarrhea cases. The thermolable haemolysi (*tlh*) gene, as well as the gastroenteritis related pathogenicity genes *tdh* and *trh*, are recognized as useful markers for the detection of *V. parahaemolyticus*. Conventional PCR assay need a specific thermal cycler and possesses a relative low sensitivity [8,16,49]. At present, loop-mediated isothermal amplification (LAMP) have been broadly used as a rapid detection method [20–25].

In the current study, a simple and rapid PMA-LAMP assay on the detection of *V. parahemolyticus* has been developed and evaluated, with further application on food-borne *V. parahemolyticus* strains.

2. Materials and methods

2.1. Strains and bacterial culturing

V. parahemolyticus strain CGMCC1.1614 and other three *V. parahemolyticus* strains (V01, $tlh^{-}trh^{+}tdh^{-}$; V02, $tlh^{-}trh^{-}tdh^{+}$ and V03, $tlh^{+}trh^{-}tdh^{-}$) were included for the development and evaluation of the PMA-LAMP assay. The strains were isolated from various food samples during 2003–2007 and stored in −80 °C. Bacterial incubation was performed at 28 °C in Luria-Bertani (LB) broth with shaking at 120 rpm in a Biosafety Level 2 (BSL-2) biological safety cabinet. PCR was previously performed to amplify the *tlh*, *tdh* and *trh* gene to identify the serotype [48].

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2.2. VBNC induction and determination

The VBNC state induction were performed as described previously [36,37]. In brief, *V. parahaemolyticus* strains was inoculated into 100 ml LB (3.0% NaCl, pH 7.5) broth, followed by an overnight cultivation at 28 °C using a shaker incubator in 120 rpm. The *V. parahaemolyticus* cell suspension was cultured at 4 °C for 50 d for induction of VBNC state. The VBNC state was identified by the CFU testing and microscopical DVC. For the counting, bacteria cells were streaked onto nutrient agar and incubated at 28 °C.

2.3. Template DNA preparation and LAMP assay

Genomic DNA of *V. parahaemolyticus* strains for LAMP amplification was extracted from cell suspension cultivated overnight in LB culture [11–15]. Total DNA extraction was performed according to the instruction of the kits (Dongsheng Biotech, Guangzhou). In brief, 2 ml culture at logarithmic growth phase was added to a 1.5 ml micro centrifuge tube and centrifuged at 12,000 rpm for 2 min to pellet the cells. After removing the supernatant, 180 μl of buffer (20 mM Tris, pH 8.0; 0.2 mM EDTA, 1.2% Trition 100) and 20 mg/ml lysozyme were added by vortexing. The culture was incubated at 37 °C for 60 min. Then, 4 μl RNase A (100 mg/ml) was added and incubated for 5 min at room temperature. The proteinase K of 20 μl and lysate MS of 220 μl were added and incubated at 65 °C for 10 min to yield a homogeneous solution. Following the addition of 220 μl absolute ethanol, the whole culture was pipeted into the spin column placed in a 2 ml collection tube and centrifuged at 12,000 rpm for 1 min. After the discard of flow-through, the culture was scoured by wash buffer PS and wash buffer PE, centrifuged for 1 min at 12,000 rpm saving the precipitation. The spin column was placed in a clean 1.5 ml microcentrifuge tube, and 30–100 μl Eluent Buffer AE (pre-warmed to 65 °C) was pipeted directly. The culture was incubated at room temperature for 2 min, and then was centrifuged for 2 min at 12,000 rpm for elution. The tube contains the purified DNA. The processed DNA was stored at −20 °C for future use [17–19,26–34].

The LAMP protocol was designed to detect conservative sequence for specific *tlh*, *tdh* and *trh* gene of *V. parahaemolyticus* [42,43] via sequence alignment and Primer Explorer V4 software (http://primerexplorer.jp/e). For each of the target genes, a set of inner primers (including forward and backward inner primer), outer primers (including F3 and B3) and loop primer were designed for LAMP to target 6 distinct regions (with primer sequences listed in Table 1). To ascertain the detection limits of LAMP assay, template DNA from *V. parahaemolyticus* CGMCC1.1614 was diluted for serial 10-fold. LAMP is carried out in a total of 25 μl reaction mixture containing 1.6 μM (each) of the primers FIP and BIP, 0.2 μM (each) of the primers F3 and B3, 1.6 mM of deoxynucleoside triphosphates, 6 mM $MgSO_4$, 1 M betaine (Sigma, St. Louis, MO, USA), 1 X thermopol buffer (New England Biolabs, Ipswich, MA, USA), and the specified amounts of target genomic DNA. The reaction was heated at 95 °C for 3 min, then chilled on ice, 1 μl (8 U) of Bst DNA polymerase (New England Biolabs, Ipswich, MA, USA) was added, after incubation at 65 °C for 60 min, the reaction was terminated by heating at 80 °C for 2 min. LAMP amplification determination had also been dyed with Sybr Green, positive or negative were determined through both visually observation of the color change and a UV-fluorescence assay. Experiment was performed in triplicate to ensure reproducibility.

2.4. The establishment and evaluation of PMA-LAMP

The PMA processing were performed as describes previously [38]. In the current study, PMA was replaced by EMA to enable crosslinking with bacterial DNA molecule for better performance [40,41] In brief, 0.1 ml cell suspensions in VBNC state or dead state containing a total of 1.35×10^7 CFU were treated by a series of volume of PMA stock solution (0.1 μg/μl) and then removed immediately into a dark room at room temperature for 5 min, which were then set into crushed ice with their lids off, followed by an exposure to the halogen lamp (500 W) for 15 min at a distance of 15 cm.

The LAMP assay was comprised by 1 μl of template DNA, 1 μl of Bst DNA polymerase and 40 pmol each FIP and BIP, 5 pmol each F3 and B3, 1 M betaine, 6 M $MgSO_4$, 1.6 mM dNTPs and ddH_2O [44]. The final volume of PMA-LAMP assay was adjusted to 25 μl. The amplification conditions were followed by incubation at 65 °C for 90 min and heating at 80 °C for 2 min to terminate the reaction. The amplification conditions were followed by incubation at 65 °C for 60 min and heating at 80 °C for 2 min to terminate the reaction. The products were analyzed by electrophoresis in 2.0% agarose gel and SYBR Green I method [45,46].

2.5. The application of PMA-LAMP

In addition to the strain CGMCC1.1614, other three *V. parahemolyticus* strains (V01, $tlh^-trh^+tdh^-$; V02, $tlh^-trh^-tdh^+$ and V03, $tlh^+trh^-tdh^-$) were further applied for PMA-LAMP as aforementioned, with determination by eye observation of SYBR Green I.

3. Results

3.1. Survival curve in cold oligotrophic state

V. parahaemolyticus were cultured in cold oligotrophic (4 °C) state at a cell density of 2.10×10^9, and the number of bacteria was detected every 5 d by DVC and plate count method (Fig. 1). The number of viable cells determined by DVC method was decreased to some extent, but the number of bacteria determined by plate count method was significantly decreased. In the first 50 d, the number of viable cells was 10^7, but the number of culturable bacteria was zero (Fig. 2), which means most of *V. parahaemolytiucs* were in VBNC state.

3.2. The inhibition of PMA for LAMP in VBNC state and heat-killed cells

The dead cell suspensions (1.0×10^7 CFU/ml) were treated with PMA by a series of concentrations of 0 μg/ml, 0.8 μg/ml, 1.6 μg/ml, 2.0 μg/ml, 4.0 μg/ml, 6.0 μg/ml, 8.0 μg/ml, 10.0 μg/ml, 12.0 μg/ml, 14.0 μg/ml, 16.0 μg/ml, 18.0 μg/ml, 20.0 μg/ml, and 25 μg/ml. The amplification of target DNA derived from heat killed cells was completely inhibited when such cells were treated with PMA at a concentration of 20 μg/ml (Fig. 3). In contrast, target DNA from heat killed cells was amplified when the PMA concentration was 0.8–18 μg/ml. For 10^7 CFU/ml bacterial cells, 20 μg/ml PMA is therefore suitable for discrimination of DNA from cells in VBNC state and in dead state by the LAMP assay.

3.3. Evaluation of PMA-LAMP

For extraction of the genomic DNA from *V. parahaemolyticus* [50], the concentration of DNA is 903 ng/μl. DNA stock solution was diluted from 10^{-1} to 10^{-9} by ten-fold dilution. Then the DNA dilution solutions were used as templates in the LAMP and PCR assays. The detection limits of LAMP and PCR assays for genomic DNA were found to be 9 fg/tube and 900 pg/tube (Data not shown). The comparative sensitivity of LAMP and PCR indicated that LAMP was much more sensitive than PCR. For LAMP products, When SYBR