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Specific Detection of *Vibrio Parahaemolyticus* in Viable but Non-Culturable State by EMA-LAMP Technique

Li Wang, Qingping Zhong[+] and Zhenlin Liao

College of food science, South China Agricultural University, Guangzhou, 510642, China

Abstract. The viable but non-culturable (VBNC) state of *Vibrio parahaemolyticus* cannot be detected by traditional culturing methods while remain potentially pathogenic activity under favourable conditions. Loop-mediated isothermal amplification (LAMP) combined with the ethidium monoazide (EMA) treatment was applied for differentiation *V. parahaemolyticus* cells in VBNC state from that of dead state. 40 μg/ml EMA is ideally suitable for discrimination of DNA in VBNC state and dead state from food by the EMA-LAMP assay. The *tlh* gene can be amplified remarkably within 1h when the amount of DNA was 9 fg. The procedure offers great potential and merits further and comprehensive field validation before it can become part of routine surveillance-response approaches in China or elsewhere.

Keywords: viable but non-culturable (VBNC) state, *Vibrio parahaemolyticus*, EMA, Loop-mediated isothermal amplification.

1. Introduction

A pathogenic halophile, *V. parahaemolyticus*, has been well known as the causative agent of the most prevalent food poisoning [1]. This pathogen, like other members of the genus *Vibro*, is a kind of gram-negative bacillus distributed worldwide in the estuarine environment. The infection of *V. parahaemolyticus* was caused by the cross contamination of food from seafood processing, transportation and sales processes, which in humans may lead to severe watery diarrhea, vomiting and intestines convulsion, even serious gastroenteritis sepsis [2]. Since 1982, the concept of viable but non-culturable state (VBNC) of bacteria was first introduced. VBNC bacteria are those which are alive but do not give rise to visible growth under nonselective growth conditions, such as the changing of temperature, absence of nutrition, environmental pollution, ultraviolet radiation and so on. The presence of VBNC cells poses a major public health problem since they cannot be detected by traditional culturing methods and the cells remain potentially pathogenic under favourable conditions [3]. These facts have prompted interest in the development of rapid, sensitive methods for detecting this organism in food.

Currently, aiming at addressing DNA amplification for detecting the bacteria at the physiological and genetic levels, the PCR and real-time PCR methods are most commonly used for gene amplification; Although PCR assays provide more rapid identification of bacteria than conventional biochemical-based assays, PCR assay need special device. Real-time PCR assays are rapider than conventional PCR assays due to the detection of fluorescence from amplification. Real-time PCR assay is, however, not routinely used due to the requirement for an expensive thermal cycle with a fluorescence detector. Notomi and his team developed a novel method named loop-mediated isothermal amplification (LAMP), which can amplify DNA in less than an hour under isothermal conditions and with great specificity [4].

Ethidium monoazide (EMA), a kind of fluorescent dye, has recently been used as a DNA binding agent to differentiate viable and dead bacterial cells [5]. EMA has the ability to penetrate the dead cells to

[+] Corresponding author. Tel.: +86 13535121218; fax: +86-20-85280270.
E-mail address: zhongqp@scau.edu.cn.

intercalate into DNA, but not the viable ones. As a result, it could effectively inhibit DNA amplification derived from dead cells, while not affecting the amplification of DNA from the viable cells. In this research, we use EMA-LAMP method for the detection of *V. parahaemolyticus* in VBNC state and dead state.

2. Materials and Methods

2.1. Bacterial strains

One *V. parahaemolyticus* strain, CGMCC1.1614, was purchased from China General Microbiological Culture Collection Center.

2.2. Storage and culture conditions

Inoculate a loopful of bacteria into 100ml Luria-Bertani (LB) (3.0% NaCl, pH7.5) broth. Then the broth was cultured overnight at 28℃ using a shaker incubator set at 120r/min. Thereafter, store the enrichment broth in 4℃ refrigerator for 50 days at a cell density of 2.10×10^9CFU/ml. Samples were taken for viable count determination by the plate count method and determination of the VBNC state was achieved by microscopical direct viable count (DVC). For the counts, *V. parahaemolyticus* was spread onto nutrient agar, incubated at 28℃, and the number of bacterial CFU was recorded. When the number of bacterial was zero, *V. parahaemolyticus* goes into VBNC state.

2.3. Heat treatment of bacterial cells

Microcentrifuge tubes (1.5ml) containing 0.1ml of cell suspensions (1.35×10^8CFU/ml) were heated at 100℃ for 5min in a water bath. The treated tubes were cooled to room temperature and the absence of viable cells were detected by plate count method followed by incubating at 28℃ for 3 days [6].

2.4. EMA treatment for cells in VBNC state or dead cells

The 0.1ml cell suspensions in VBNC state or dead state containing a total of 1.35×10^7 CFU in microcentrifuge tubes were treated by a series of volume of EMA stock solution (0.1μg/μl) [7]. Then the microcentrifuge tubes were removed immediately into a dark room at room temperature for 5min. After that, the tubes were set into crushed ice with their lids off, and exposed to the halogen lamp (500W) for 15min, at a distance of 15 cm.

2.5. Lysing of bacterial cells for LAMP and PCR

To obtain the DNA of the cells in VBNC state and dead state, those which were treated by EMA, DNA template was performed following the reference published earlier [8]. After EMA treatment, take 0.5ml enrichment broth into 1.5ml microcentrifuge tube. Then the cells pellets were collected by centrifuging the enrichment broth at 10000r/min for 5min at 4℃. The supernatants were discarded and the cells resuspended in 0.5ml of saline-magnesium solution. The cell lysing solution designated TZ consisted of 2% Triton X-100 and 2.5mg/ml sodium azide in 0.1M Tris-HCl buffer at pH 8.0.EMA-treated cell suspensions (0.5ml) were mixed with 1.0ml TZ in 1.5ml microcentrifuge tubes followed by mixing, cells were then lysed by heating at 100℃ for 10min in a boiling water bath. Then this mixture was centrifuged in the same conditions as above to pellet cells debris, the supernatant was used in the EMA-LAMP assay and PCR.

2.6. EMA-LAMP

All primers were designed from sequence data submitted to GenBank (thermolabile hemolysin gene, tlh, M36347). The EMA-LAMP assay was comprised by 1μl of template DNA, 1μl of Bst DNA polymerase and 40pmol each FIP and BIP, 5pmol each F3 and B3, 1M betaine, 6M $MgSO_4$, 1.6mM dNTPs and ddH_2O . The final volume of EMA-LAMP assay was adjusted to 25μl. The amplification conditions were followed by incubation at 65℃ for 90min and heating at 80℃ for 2min to terminate the reaction. The products were analyzed by electrophoresis in 2.0% agarose gel and SYBR Green I.

2.7. PCR reaction

The PCR reaction was performed with two outer primers. The PCR mixture consisted of 40mmol/l MgCl2, 10μmol/l of each primers, 2.5μl of 2.5mmol/l dNTPs, 2.5μl of 10×PCR buffer, 14μl ddH_2O, and 5U/μl Taq

polymerase for a volume of 24μl mix. So 1μl DNA template was analyzed in a final volume of 25μl. The cycle conditions were: 94℃ for 3min, followed by 30 cycles of denaturation at 94℃ for 1min, annealing at 58℃ for 30s and 72℃ for 30s, and a final extension at 72℃ for 10min. The PCR products were analyzed by electrophoresis in 2% agarose gel.

3. Results

3.1. The survival curve of *V. parahaemolyticus* in cold oligotrophic state

V. parahaemolyticus were cultured in cold oligotrophic (4℃) state at a cell density of 2.10×10^9, and the number of bacteria was detected every 5 days by DVC and plate count method. 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 days, the number of viable cells was 10^7, but the number of bacteria can be cultured was zero (Fig.1), which means most of *V.parahaemolyticus* went into the VBNC state.

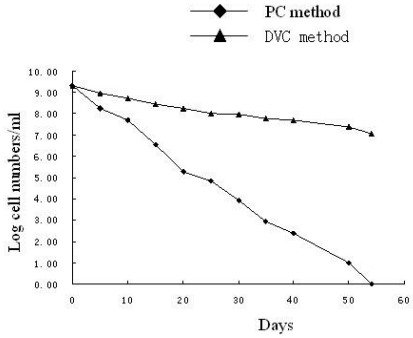

Fig. 1: Survival curves of *V. parahaemolytices* in sterilized aged seawater microcosms incubated at 4℃

3.2. The morphological features of *V.parahaemolyticus* under the fluorescence microscope and scanning electron microscope

Fresh bacteria, the cells in VBNC state and dead cells were treated with Apoptosis Detection Kit (called KGA-501) produced by Keygentec company in China. The bacteria were in normal state and in VBNC state showed green fluorescence under flurescence microscope, but dead cells showed red fluorescence under flurescence microscope (Fig.2). At the same time, for fresh bacteria and the cells in VBNC state, the morphological features of cells were observed under scanning electron microscope (Fig. 3).

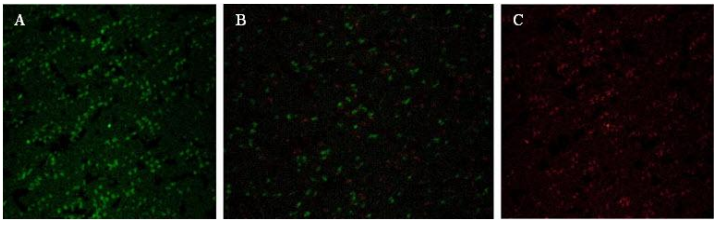

Fig. 2 Morphological characteristics of *V. parahaemolytices* in different states under fluorescence microscope, A. the normal cells; B. the VBNC cells; C. the dead cells.

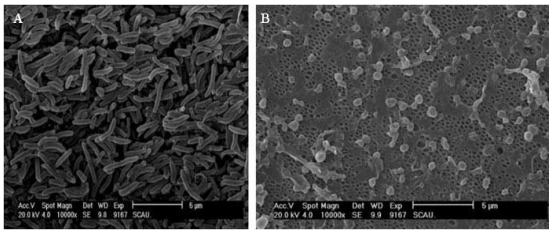

Fig. 3 Morphological characteristics of different state of *V. parahaemolytices* under scanning electron microscope, A. the normal cells; B. the VBNC cells (Scale bar=5μm)

3.3. Sensitivity of LAMP and PCR

The cells in VBNC state were treated with EMA by a series of concentrations, 20 μg/ml EMA is therefore suitable for discrimination of DNA from cells in VBNC state and in dead state by the EMA-LAMP assay. The dead cell suspensions (2.1×10^9 CFU/ml) were treated with EMA by a series of concentrations, 40 μg/ml EMA is ideally suitable for discrimination of DNA from *V. parahaemolyticus* in VBNC state and dead state from food by the EMA-LAMP assay. For extraction of the genomic DNA from *V. parahaemolyticus*, 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 (Fig.4A and Fig. 4B). The comparative sensitivity of LAMP and PCR indicated that LAMP was much more sensitive than PCR. For LAMP products, when SYBR Green I was added, all the amplification products examined positive by electrophoresis turned green, whereas all the reaction products that were negative analyzed remained orange (Fig. 5). Thus the sensitivity of the LAMP can simply be judged by the naked eye, and the results were in well consistency with the detection by electrophoresis. The LAMP assay showed a higher sensitivity with PCR assay in shorter time.

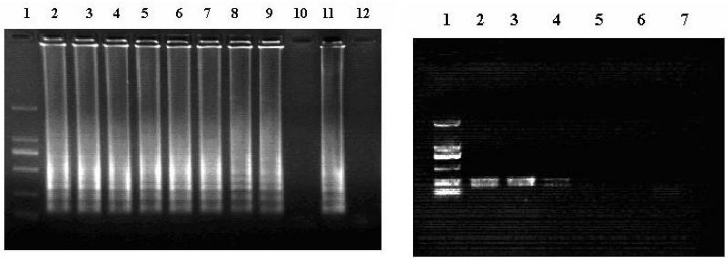

Fig. 4 Detection limits of LAMP assay and PCR assay for genomic DNA derived from *V. parahaemolyticus*
Lane 1, 2000bp marker; lane 2, 90ng; lane 3, 9ng; lane 4,900pg; lane 5, 90pg; lane 6, 9pg; lane 7, 900fg; lane 8, 90fg; lane 9 9fg; lane 10, 0.9fg; lane 11, positive control; lane 12, negative control (left). Lane 1, 2000bp marker; lane 2, 90ng; lane 3, 9ng; lane 4, 900pg; lane 5, 90pg; lane 6 9pg; lane 7, negative control (right).

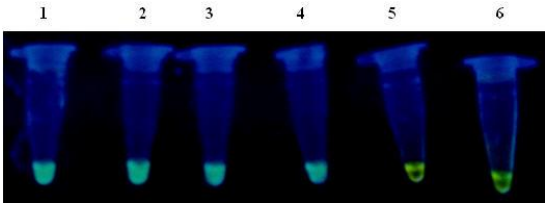

Fig. 5 The detection of LAMP products by SYBR Green I. The solutions turned green in the presence of LAMP products or remained orange in the absence of amplification. Tube 1-4, positive samples; Tube 5-6, negative samples.

4. Discussion

As pathogens remains one of the leading concerns for public health [9], the potential VBNC state of *V. parahaemolyticus* posed an obstacle for the conventional CFU testing method, which is not capable of detecting *V. parahaemolyticus* in VBNC state. Despite a number of counting methods established and documented, including AODC and DFA [10], [11], the present study is the first report on the detection for *V. parahaemolyticus* in VBNC state with a DNA-amplification based methodology. Regular PCR has been commonly used with the advantage of high sensitivity and specificity; nevertheless, it is unable to differentiate the viable and dead cells. Despite the introduction of EMA-PCR and EMA-real time PCR have been reported in occasional report [12], [13], however, the requirement of post detection procedures, the risk of contamination, as well as the inability to detect low levels of pathogenic *V. parahaemolyticus* bacteria in the presence of a high background of nonpathogenic *V. parahaemolyticus* organism for regular PCR, as well as the limitation included the requirement for trained personnel, operating space, expensive equipment and reagents for real-time PCR, had restricted its application.

Capable of penetrating into those cells with undergone lethal membrane damage, EMA has been considered to be a potential strategy for distinguishing viable and dead bacterial cells. In this current study, an EMA-LAMP detection assay had been developed and evaluated for the diagnostic detection of *V. parahaemolyticus* in VBNC state. LAMP amplifies DNA with high efficiency under isothermal condition without a significant influence of the presence of non-target DNA, which is advantageous on the low detection limit compared with PCR, with only a few copies of DNA. Its reaction could be finished in a water bath or heat block in a regular laboratory. The products were detected by electrophoresis in 2.0% agarose gel and SYBR Green I method, which were easy and simple.

In conclusion, this improved EMA-LAMP assay was demonstrated to be a useful and powerful tool for rapid detection of *V. parahaemolyticus* strains. We confirmed that using of EMA-LAMP method, there is no notable inhibition for DNA amplification derived from *V. parahaemolyticus* in VBNC state. But 20 μg/ml EMA inhibited the DNA amplification derived from dead cell suspensions (10^7CFU/ml). When the concentration of EMA was 40 μg/ml, DNA amplification was inhibited derived from dead cell suspensions (10^9 CFU/ml). 40 μl/ml EMA as the optimized concentration for EMA-LAMP for distinguishing *V. parahaemolyticus* in VBNC state from dead state. Undoubtedly, rapidness, easiness and cost-effectiveness of EMA-LAMP assay will aid in the broad application of bacteriological detection of *V. parahaemolyticus* in VBNC state.

5. Acknowledgements

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6. References

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