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The novel loop-mediated isothermal amplification based confirmation methodology on the bacteria in Viable but Non-Culturable (VBNC) state

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ABSTRACT

As a self-protection mechanism, the viable but non-culturable (VBNC) state provides the ability against conventional detection methods among various foodborne pathogens. The ability of forming colonies is lost while metabolism is still maintaining in VBNC state cells. Recently, ethidium monoazide (EMA) and propidium monoazide (PMA) have been widely applied on the detection of foodborne pathogens in VBNC state. Combined with loop-mediated isothermal amplification (LAMP), the PMA/EMA-LAMP showed a significant priority in high sensitivity, specificity and rapidity over conventional PCR based assays. Particularly, PMA/EMA-LAMP has been proved as an effective method in the detection of *Escherichia coli*, *Vibrio parahaemolyticus* and *Staphylococcus* in VBNC state. Based on the current investigations, the VBNC mechanism and current detection method for VBNC-state foodborne pathogens were introduced and discussed in this review.

Contents

1. Introduction

As a global concern on food safety, foodborne pathogens has caused a significant threat to public health during the past decades. Remarkably, more than one factors that resulted in food safety incidents, such as additives, foodborne bacteria, heavy metal pollution and drug residue, among which bacteria account for over 31% [1–3]. Moreover, the investigation conducted by WHO indicated

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that the infections by virulence in foodborne pathogens was at an upward tendency. The Viable but Non-Culturable (VBNC) state is a unique survival strategy for the bacteria against adverse environment. Bacteria in VBNC state cannot grow on the heterotrophic plate but still possess metabolic activity, pathogenicity and toxicity. Bacteria in VBNC state is capable of recovering and restoring for cultivation and pathogenicity when in suitable condition. The emergence of foodborne pathogens in VBNC state promoted the occurrence of foodborne infections [4–7]. Particularly, it has been demonstrated that the expression of *rfbE* of *Escherichia coli* (*E. coli*) O157 cell is higher in VBNC state, as well as relatively lower expression of *stx1* and *stx2* genes compared with normal cells [8].

To date, foodborne bacteria are usually monitored and characterized by laboratories using standard culture methods, which includes pre-enrichment, selective enrichment, followed by a series of morphological and biochemical identification. However, it takes several days (commonly in 4–7 days) to obtain the identification result [9,10]. The bacteria in VBNC state is not able to colonize on regular plate medium, thus conventional microbiological methods are no longer proper for the detection of cells in VBNC state. The effective and efficient methods are desperately in need in the surveillance of food quality and foodborne pathogenic bacteria [11,12,59–61]. Conventional methods for detection of foodborne pathogens are quite complex and time consuming, which is not meet the requirement of point-of-need (PON) diagnostics (low cost, simplicity in operation and the ability for high-throughput with quantitative analysis) [13].

Over the past decades, multiple novel methods (immunological techniques, PCR-based molecular assays and biosensor technology) has replaced the conventional detection methods, which decreased unnecessary hospitalization and associated healthcare costs [14–16]. Particularly, nucleic acid based methodology offers high sensitivity and specificity. In 2000, Notomi [17] firstly reported a novel detection method named loop-mediated isothermal amplification (LAMP). LAMP amplification employs a Bst DNA polymerase with high strand displacement activity and a set of four to six primers that recognize six distinct areas of the target sequence with high specificity. Compared with other rapid detection methods, LAMP shows priority in high specificity, sensibility, rapidity and simple operation under isothermal conditions [18–20]. Therefore, the LAMP assay has been successfully applied on the detection of common pathogens including bacteria, viruses, fungi and parasites. However, some drawbacks still exists [21,62–64]. For example, the inability of the PCR or LAMP itself to discrimination between viable and dead cells and the presence of VBNC cells result in an overestimation of the target microorganism. To overcome this drawback, PCR and LAMP were combined with viability dyes to detect the viable foodborne pathogens directly [22–24].

In recent years, DNA-intercalating agents have been employed to prohibit the DNA amplification from the dead bacteria. EMA and PMA can penetrate membrane-damaged cells and bind to DNA molecule, thus inhibiting DNA amplification of dead cells, and allow the amplification of unbound DNA from viable cells at the same. Consequently, these dyes were combined with PCR or LAMP to differentiate VBNC cells from dead cells [25,26]. This review provides an oversight on research progress and LAMP-based methods of VBNC state.

2. The mechanism of VBNC state

The VBNC state was firstly reported in 1982. To date, over 85 species of bacteria was proved to be induced into VBNC state, including *E. coli*, *Licteria monocytogenes*, *Salmonella*, *Shigella dysenteria*, *Vibrio vulnificus* and *Lactobacillus* [27–31]. The mechanism of VBNC state, however, remains unclear. Two hypotheses about this topic was introduced by Diane and Nystrom [32]. The absence of nutrition resulted in cytoplasmic degradation decreased the cell viability, leading to the weakness and presence of cell apoptosis. The reversibility of VBNC cells depends on the degree of cell injury. In other words, the bacteria will eventually die once the cells are excessively damaged. Another hypothesis is that the VBNC state is an adaptive strategy of nonsporulating bacteria against adverse environment (exposure to extreme temperature, UV light shock, heavy metals, low pH, absence of nutrition and oxidative stress). Cells maintains metabolic activity under VBNC state. After entering the VBNC state, the cells are not damaged while maintaining their metabolic activity, The induction mechanism is similar to the spore formation. Nowadays, the latter hypothesis was accepted.

3. Current methodologies for detection of bacteria in VBNC state

A variety of phenotype-based methods has been applied in the screening of VBNC state, including fluorescent antibody, redox-based respiration detection and Live/Dead kit. Moreover, some genetic tools (such as gene probes, molecular hybridization and RT-PCR) were designed for the rapid screening. *E. coli O157:H7* in VBNC state was successfully screened with RT-PCR targeting on specific genes as *16S rRNA*, *MobA*, *rfbE* and *stx1* [33]. Besides, the application of fluorescence visualization technique enabled the directly detection of *V. parahaemolyticus* in the VBNC state in nature, laying significant impact on the detection of VBNC state pathogens in mixed environmental samples [34,67]. Modern instrument analytical technique is another method (through the combination of matrix-assisted laser desorption/ionization time of flight mass spectrometry and multivariate mass spectrometry.) adapted by Kuehl et al. for the detection of *Enterococcus faecalis* in VBNC state based on different physiological status [35]. In recent years, some researchers devoted to the study of biological sensors to identify the VBNC bacteria with the nucleic acid as the recognition element, as much significant advance have been made for the presence of aptasensor and genosensor [36]. However, the reliability of biosensor needs further investigation as they were rarely applied on the identification of bacteria in VBNC state.

4. PMA/EMA-LAMP

PMA/EMA-LAMP employed the optimized primers to explorer the levels and proportions of different components for the thre3 kinds of foodborne pathogens in VBNC state [37]. Detailed procedure of PMA/EMA-LAMP are as follows.

Firstly, 0.1 ml cell suspensions reached 10^7 CFU were treated with certain concentration of PMA or EMA stock solution and then remove immediately into a dark room for 5min (at a room temperature), 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. Secondly, total DNA extraction of VBNC and dead cells was performed according to the instruction of the common genomic DNA extraction kits, followed by the LAMP reaction described previously. Heating and isothermal amplification were performed on a heating block and water bath. Finally, the result was confirmed either the color change, turbidity, agarose gel analysis or real time fluorescence, which are similar with conventional LAMP.