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Comparison between digital PCR and real-time PCR in detection of *Salmonella typhimurium* in milk

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ABSTRACT

As a kind of zero-tolerance foodborne pathogens, *Salmonella typhimurium* poses a great threat to quality of food products and public health. Hence, rapid and efficient approaches to identify *Salmonella typhimurium* are urgently needed. Combined with PCR and fluorescence technique, real-time PCR (qPCR) and digital PCR (ddPCR) are regarded as suitable tools for detecting foodborne pathogens. To compare the effect between qPCR and ddPCR in detecting *Salmonella typhimurium*, a series of nucleic acid, pure strain culture and spiking milk samples were applied and the resistance to inhibitors referred in this article as well. Compared with qPCR, ddPCR exhibited more sensitive (10^{-4} ng/μl or 10^{2} cfu/ml) and less pre-culturing time (saving 2 h). Moreover, ddPCR had stronger resistance to inhibitors than qPCR, yet absolute quantification hardly performed when target's concentration over 1 ng/μl or 10^{6} cfu/ml. This study provides an alternative strategy in detecting foodborne *Salmonella typhimurium*.

1. Introduction

Since infectious disease of foodborne caused a major crisis on human health, attentions have been given to rapid and accurate detections of causative pathogens in food products. Among all foodborne pathogens, *Salmonella typhimurium* is treated as a common bacterium causing foodborne illnesses around the world (Kirk et al., 2015). In China, regarded as the second major foodborne pathogen, *Salmonella* species were related to about 40% incidents of bacteria-related food poisoning (Chen et al., 2010; Xiao et al., 2015). The majority of people infected with *Salmonella typhimurium* suffer from diarrhea, fever, abdominal cramps and some severe patients need to be hospitalized. Possessing dramatically food reservoir, *Salmonella typhimurium* was associated with outbreaks of illnesses linked to contaminated cucumber, chicken, raw tuna, beef, pork, milk, egg, seafood and many other foods (Omiccioli et al., 2009). Hence, a reliable method detecting foodborne *Salmonella typhimurium* plays a crucial role in guaranteeing food safety and citizens' health.

The traditional method to detect *Salmonella typhimurium* is based on culture medium including pre-enrichment, selective culturing, isolation on selective medium, calculating positive colonies by biochemical and serological analysis (Schönenbrücher et al., 2008). Be deemed to time consuming and laborious, 3–6 days are requisite to complete the whole process (Wang et al., 2017). Furthermore, the traditional method showed a handicap in sensitivity of detecting foodborne pathogens, especially for those regarded as zero tolerance (Settanni and Corsetti, 2007). To overcome these drawbacks, PCR methods based on nucleic acid amplification tests (NAATs) are widely applied in detecting foodborne *Salmonella* species. Several articles demonstrated the effect of end-point PCR methods targeting different genes of *Salmonella typhimurium* in food products (Bansal et al., 2006; Kim et al., 2006; Maciorowski et al., 2008; Naravaneni, 2005; Radji et al., 2010). Thus, be subjected to ager analysis, end-point PCR method has obstacles in quantitative analysis (Borowsky et al., 2007).

Relying on calculating increment of fluorescence generated by amplified templates instead of agar gel analysis, abbreviating the time-consuming to 3 days or less (Corless et al., 2001) makes qPCR be widely applied in foodborne pathogens' measurement (Elizaquível et al., 2012; O'Regan et al., 2008; Zhang et al., 2011). Multiplex assays, minor grove binding (MGB) probe and propidium monoazide (PMA) applied in

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qPCR made it more flexible in practical application (Chen et al., 2010; Nocker et al., 2007; O'Regan et al., 2008). Owning to its superiority, qPCR becomes so common in detecting foodborne *Salmonella typhimurium* (Jung et al., 2005; Zheng et al., 2014), but limitations existing in measuring trace sample or absolute quantification.

As the latest generation of PCR and one of the most robust methods in molecular quantification, several articles in recent years have reported ddPCR in the field of food and drug testing, bacteria and viruses monitoring, gene modification and clinical diagnosis research (Cai et al., 2014; Day et al., 2013; Klančnik et al., 2015; Morisset et al., 2013; Porcellato et al., 2016). Differing from the traditional notion of ddPCR relying on multi-well plates, commercial ddPCR platforms developed droplets or silicon substrate approaches running tens of thousands of individual reactions in parallel. After PCR amplification and fluorescence signal collection, initial concentration of the target is calculated according to the pattern of Poisson distribution (Hindson et al., 2013). Compared with qPCR, ddPCR has advances in performing absolute quantification procedures without a standard, rapid data analysis, sensitivity, precision and resistance to inhibitors (Whale et al., 2012). Recent articles have demonstrated the sensitivity and precision of ddPCR in the quantitative detection of bacteria in sediment and commercial poultry processing water samples, yet its application for the detection of *Salmonella typhimurium* in food samples has so far not directly been defined (Rothrock et al., 2013; Singh et al., 2017).

To our knowledge, several methods have related to qPCR in detecting foodborne *Salmonella* spp. (Almeida et al., 2013; Tatavarthy et al., 2017) but rarely to ddPCR. Hence, whether ddPCR having advances in detecting and quantifying *Salmonella typhimurium* in milk was measured. Our results showed ddPCR exhibited stronger sensitivity and resistant to PCR inhibitors. Moreover, as a significant carrier of transmission of pathogenic bacteria, aseptic milk with *Salmonella typhimurium* culture was prepared to evaluate the efficacy of both systems, and the results manifested that ddPCR maintained lower limit of detection (LOD) and less enrichment time.

2. Materials and methods

2.1. Bacterial strains and culturing conditions

Salmonella typhimurium and other control strains applied in this study were obtained from Shandong Institute for Food and Drug Control (Table 1). All tested strains were preserved in lysogeny broth media (Beijing Land Bridge Technology Co., Ltd.) at − 20 °C, activated in nutrient agar media (Beijing Land Bridge Technology Co., Ltd) for 24 h at 37 °C and enriched in nutrient broth media (Beijing Land Bridge Technology Co., Ltd) for 24 h at 37 °C. The number of *Salmonella typhimurium* suspension was calculated by plate count agar (PCA, Beijing Land Bridge Technology Co., Ltd).

2.2. DNA extraction

For all tested strains, 60 °C water bath for 15 min to promote bacterial cells lysis. Genomic DNA of *Salmonella typhimurium* was extracted by UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories) following manufacturer's instruction. Quality control of extracted genomic DNA samples was performed by Nanodrop 2000 (Thermo Fisher Scientific). Then, a series of 10-fold dilution DNA (concentration ranging from 10 ng/μl to 10^{-4} ng/μl) were prepared, and each gradient DNA sample was analyzed by qPCR and ddPCR (Fig. 1).

Table 1
List of bacterial species applied in specificity test.

Species	Origin	Strain number[a]	Results[b]
Salmonella typhimurium	Unknown	CICC 21483	+
Listeria monocytogenes	Unknown	ATCC 19111	−
Vibrio parahaemolyticus	Unknown	CICC 10552	−
Escherichia coli	Unknown	ATCC 25922	−
Enterobacter sakazakii	Unknown	CICC 21544	−
Micrococcus luteus	Unknown	ATCC 10240	−

[a] CICC: China Center of Industrial Culture Collection, ATCC: American Type Culture Collection.
[b] +: positive amplification, −: negative amplification.

2.3. Primer and probe

Based on FimY gene of *Salmonella* spp., a pair of primers and probe were selected from China food safety standard (CFDA.SN/T 1870 - 2016). The forward primer 5′-GCGGCGTTGGAGAGTGATA-3′, the reverse primer 5′-AGCAATGGAAAAAGCAGGATG-3′ and the taqman probe 5′-FAM-CATTTCTTAAACGGCGGTGTCTTTCCCT-MGB-3′ were purchased from Sangon (Sangon Biotech Co., Ltd.). Specificity of the primer pair and probe was measured by several pathogenic bacteria and blank control (Table 1).

2.4. qPCR assays and procedure

For each reaction, 1 μl DNA, 10 μl Premix Ex Taq (Takara Biotechnology Co., Ltd.), 1 μl of 10 μM forward and reverse primer, 0.8 μl of 10 μM probe, 0.4 μl Rox Reference Dye (Takara Biotechnology Co., Ltd.) and 6.8 μl nuclease-free water (Ambion, Thermo Fisher Scientific) were added to total volume of 20 μl. QuantStudio 5 Real-time PCR System (Life Technologies Inc.) was applied in thermal cycling: 50 °C hold for 2 min, followed by 95 °C for 30 s, then 40 cycles consisting of 95 °C for 5 s and 60 °C for 30 s per cycle, and finally cooled to 4 °C for 2 min. The output data were analyzed by associated software.

2.5. ddPCR assays and procedure

For each reaction, 1 μl DNA, 7.5 μl QuantStudio 3D Digital PCR Master Mix V2 (Life Technologies Corporation), 1 μl of 10 μM forward and reverse primer, 0.3 μl of 10 μM probe, 5 μl nuclease-free water were added to total volume of 14.8 μl. QuantStudio 3D Digital PCR System was used in thermal cycling: 96 °C hold for 10 min, followed by 39 cycles consisting of 56 °C for 5 s and 98 °C for 30 s, then a holding stage followed at 60 °C for 2 min. The output data were analyzed by associated software.

2.6. PCR inhibitor dilution series

Common PCR inhibition existing in milk (Schrader et al., 2012) or DNA extracted approaches, calcium ion and ethanol were added in PCR reaction with different DNA concentration. Diluted calcium chloride powder (Sigma Aldrich) in nuclease-free water or ethanol (Sinopharm Chemical Reagent Co., Ltd) was added, and the final concentration ranging from 4 mM to 6 mM or 0.5% to 2.5%. Then, each reaction performed qPCR and ddPCR procedure as described above.

2.7. Spiking sample

To prepare spiking milk samples, 1 ml of each concentration gradient of *Salmonella typhimurium* pure culture added in 24 ml sterile milk. Genomic DNA extracted by UltraClean Microbial DNA Isolation Kit from each spiking milk sample was analyzed by both platforms as described above (Fig. 1). In addition, 25 ml spiking milk mixed with 225 ml of buffered peptone water (BPW, Beijing Land Bridge Technology Co., Ltd) was prepared to estimate pre-cultured time-consuming (Fig. 1). Pre-enrichment process was promoted among samples out of LOD of two platforms for 2 h, 4 h, 6 h. Then, extracted DNA from each time phase was analyzed to measure time-consuming.