J. Dairy Sci. 99:1–10
http://dx.doi.org/10.3168/jds.2016-11538

A new application of a sodium deoxycholate-propidium monoazide-quantitative PCR assay for rapid and sensitive detection of viable *Cronobacter sakazakii* in powdered infant formula

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ABSTRACT

A rapid, reliable, and sensitive method for the detection of *Cronobacter sakazakii*, a common foodborne pathogen that may cause serious neonatal disease, has been developed. In this study, a rapid real-time quantitative PCR (qPCR) assay combined with sodium deoxycholate (SD) and propidium monoazide (PMA) was developed to detect *C. sakazakii* contamination in powdered infant formula (PIF). This method could eliminate the interference from dead or injured bacteria. Optimization studies indicated that SD and PMA at 0.08% (wt/vol) and 5 μg/mL, respectively, were the most appropriate. In addition, qPCR, PMA-qPCR, SD-PMA-qPCR, and plate count assays were used to account for the number of viable bacteria in cell suspensions that were exposed to a 55°C water bath at different length of time. As a result, the viable number by PMA-qPCR showed significantly higher than of the number from SD-PMA-qPCR or plate counts. The number of viable bacteria was consistent between SD-PMA-qPCR and traditional plate counts, which indicated that SD treatment could eliminate the interference from dead or injured cells. Using the optimized parameters, the limit of detection with the SD-PMA-qPCR assay was 3.3×10^2 cfu/mL and 4.4×10^2 cfu/g in pure culture and in spiked PIF, respectively. A similar detection limit of 5.6×10^2 cfu/g was obtained in the presence of the *Staphylococcus aureus* (10^7 cfu/mL). The combined SD-PMA-qPCR assay holds promise for the rapid detection of viable *C. sakazakii* in PIF.

Key words: *Cronobacter sakazakii*, sodium deoxycholate, propidium monoazide, quantitative PCR

INTRODUCTION

Cronobacter sakazakii is a member of the *Enterobacteriaceae* family; its name was changed from "yellow-pigmented *Enterobacter cloacaea*" in 1980 (Farmer et al., 1980). It belongs to a group of gram-negative rods, oxidase-negative, catalase-positive, facultative anaerobic, peritrichous, and generally motile bacteria (Iversen et al., 2008). *Cronobacter sakazakii* is a commonly reported foodborne pathogen that can cause life-threatening meningitis, sepsis, and necrotizing enterocolitis in neonates (van Acker et al., 2001; World Health Organization, 2004; Norberg et al., 2012; Hunter and Bean, 2013). Many studies reported that powdered infant formula (**PIF**) is the main route of infection for these diseases and with 40 to 80% fatality rate to newborns (Liu et al., 2006a; Norberg et al., 2012; Cai et al., 2013). The *C. sakazakii* infections in neonates has aroused wide attention all over the world. The Food and Agriculture Organization of the United Nations (**FAO**) and the World Health Organization have focused on the issue that the PIF contaminated with *C. sakazakii* and *Salmonella* has caused serious illness in infants (World Health Organization, 2004; FAO, 2008). Thus, a sensitive, rapid, and accurate detection method is urgently needed to identify *C. sakazakii* for the prevention of its transmission in PIF.

To date, various diagnostic and testing techniques have been developed for the detection of *C. sakazakii* including the widely used traditional microbiological methods that requires laborious operation and long processing time (Park et al., 2012). Alternatively, the ordinary PCR assay has been used for detection of *C. sakazakii* because of its simplicity and rapidity. Chen et al. (2013) designed primers for the sequence of the *gyrB* gene for PCR assay to rapidly detect *C. sakazakii*. Compared with conventional PCR, real-time quantitative PCR (**qPCR**) has been widely used to detect and identify *C. sakazakii* in various food products because of its high sensitivity and accuracy. In some studies,

Received May 31, 2016.
Accepted August 26, 2016.
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the target sequences in the outer membrane protein A (*ompA*) gene (Zimmermann et al., 2014), the *16S-23S rRNA* internal transcribed spacer (*ITS*; Liu et al., 2006a), the *16S rRNA* gene (Kang et al., 2007), and the *cgcA* gene (Hu et al., 2016) were used to specifically detect *C. sakazakii*. However, general qPCR could not identify viable from dead or injured cells. During the amplification phase, DNA from dead or injured cells could serve as qPCR template to generate positive signals, which could cause false-positive results. To overcome these limitations, researchers used propidium monoazide (**PMA**) combined with qPCR (**PMA-qPCR**) to remove the interference from dead cells during the amplification process of PCR (Yang et al., 2011; Xiao et al., 2015; Truchado et al., 2016). Propidium monoazide as a DNA-intercalating dye could selectively permeate through the half-baked cell membrane of dead or injured cells. Propidium monoazide could be covalently cross-linked with DNA when exposed to light to form a stable DNA modification that inhibits PCR amplification (Xiao et al., 2015). Dead or injured cells with a remaining outer membrane could serve as a protective barrier to prevent PMA permeation. As a result, some DNA from dead cells or injured cells cannot be inhibited during the PCR amplification phase (Fittipaldi et al., 2012; Nkuipou-Kenfack et al., 2013). Sodium deoxycholate (**SD**) as a detergent could disrupt cell membranes and change their permeability; thereafter, other molecules could penetrate into bacterial cells. Treatment with SD before PMA has been more effective in permeating dead cells or injured cells, which reduces the false-positive results (Yang et al., 2011; Nkuipou-Kenfack et al., 2013; Wang et al., 2014a,b).

In this study, we developed a SD-PMA-qPCR assay for rapid and accurate detection of viable *C. sakazakii* in PIF. The specific primers were designed based on the sequence of the *ITS* of *C. sakazakii* (Liu et al., 2006a,b). A SD treatment before PMA that could selectively detect viable *C. sakazakii* in pure culture was adopted into the assay. In this study, we compared the accuracy of the SD-PMA-qPCR with qPCR, PMA-qPCR, and plate count assays. To exhibit real-world application, the newly developed SD-PMA-qPCR assay for *C. sakazakii* was also applied for the detection of detect viable bacteria in PIF.

MATERIALS AND METHODS

Preparation of Bacterial Strains

The strains used in this study are listed in Table 1. *Cronobacter* strains, as well as the other bacteria strains, were cultured in Luria-Bertani (**LB**) medium in a rotary shaker at 180 rpm and 37°C. All cell pellets were suspended in sterile PBS (0.01 *M*, pH 7.4) to obtain 10 serial dilutions. The plate count method was used for enumerating viable cells that were grown on LB agar plates at 37°C for 12 h. To obtain dead cells, the cell suspensions were heated to 80°C for 5 min and the viability of the bacteria was confirmed by plating on LB agar plates for 24 h. For the optimal SD concentration studies, bacteria at 10^7 cfu/mL were heated in a water bath at 70°C, 65°C, 60°C, 55°C, and 50°C, respectively. The numbers of surviving cells were enumerated on 0.3% sodium pyruvate LB plates (0.3% LB) in an overnight culture.

Exploring Optimal SD Concentration

Earlier reports found that injured pathogenic bacterial cells lost their ability to infect hosts under normal conditions (Linder and Oliver, 1989; Morgan et al., 1993); a much more complicated and rare condition (with high and specific nutrients) is needed for recovery of injured cells to an active state, and thus injured cells could not become a hazard especially in regular conditions (e.g., food matrices). Therefore, in regular PIF matrices, injured cells could not recover to become a hazard for neonates. It is necessary to inhibit the injured cells by SD treatment. To optimize the concentration of SD for permeating the injured cells, a cell suspension (10^7 cfu/mL) was heat-treated in a water bath at 55°C for 2.5 min and immediately cooled at 4°C in an ice bath for 5 min (Arroyo et al., 2009), followed by centrifugation at 8,000 × *g* for 5 min at room temperature, and the pellet was resuspended in 500 μL of PBS, which was expected to cause a reduction in the number of viable *C. sakazakii* between 1 and 2 log units (Yang et al., 2011). Five grams of SD was prepared in a 0.1% (wt/vol) peptone water to obtain 10% (wt/vol) SD stock solution. Serial dilutions of SD from the 10% (wt/vol) stock solution were prepared by mixing with 500 μL of injured cell suspension to obtain final SD concentrations: 0, 0.02, 0.04, 0.08, 0.1, and 0.5%. These were then incubated at 37°C for 30 min in a 180 rpm rotary shaker. To recover the injured cells and enumerate the number of surviving cells, LB plates and 0.3% LB plates were prepared. Ultimately, the numbers of viable cells were determined by plate count method on an LB plate. The numbers of viable and injured cells that remained reproductive were determined by plate count method on the 0.3% LB plate. All samples were carried out in triplicate. The working principle behind the use of SD is shown in Figure 1A.