J. Dairy Sci. 101:1–9
https://doi.org/10.3168/jds.2017-13931

Development of an isothermal amplification-based assay for the rapid detection of *Cronobacter* spp.

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ABSTRACT

Cronobacter spp. is an opportunistic pathogen that is associated with rare but life-threatening neonatal infections resulting from the consumption of contaminated powdered infant formula milk (PIF). In the present study, we developed recombinase polymerase amplification (RPA) and real-time RPA for the detection of *Cronobacter* spp. in PIF for the first time by targeting the *ompA* gene. The specificity and sensitivity of the RPA and real-time RPA were validated and the practical applicability of these methods for the detection of *Cronobacter* spp. in artificially contaminated PIF samples was proved by comparing their reaction time, sensitivity, and efficacy with those of real-time PCR and the Chinese traditional method. The RPA and real-time RPA assays reduced the analysis time to less than 15 min and the results were as reliable as those of real-time PCR. Taken together, the RPA and real-time RPA assays served as fast, reliable, and sensitive techniques for the detection of *Cronobacter* spp.

Key words: *Cronobacter* spp., recombinase polymerase amplification, real-time recombinase polymerase amplification, real-time PCR

INTRODUCTION

Cronobacter spp. (formerly *Enterobacter sakazakii*) is an opportunistic pathogen associated with sporadic outbreaks of severe meningitis, sepsis, and necrotizing enterocolitis in neonates and infants with a high mortality rate of 33 to 80% (Willis and Robinson, 1988; van Acker et al., 2001; Hunter and Bean, 2013). This genus of bacteria consists of 7 species: *Cronobacter sakazakii*, *Cronobacter dublinensis*, *Cronobacter malonaticus*, *Cronobacter muytjensii*, *Cronobacter turicensis*, *Cronobacter universalis*, and *Cronobacter condimenti*. A growing number of reports have noted that *Cronobacter* spp. could be isolated from a variety of foods, and powered infant formula milk (**PIF**) was identified as a major source of *Cronobacter* spp. Infections (Muytjens et al., 1988; Drudy et al., 2006; Pina-Pérez et al., 2016). It is currently thought that even very low levels of *Cronobacter* spp. in PIF (0.36–0.66 cfu/100 g) can lead to infections in neonates (Muytjens et al., 1988; Drudy et al., 2006; Pina-Pérez et al., 2016). As the symptoms of the infection are severe, the prognosis is poor, and even low numbers of *Cronobacter* spp. pose a health risk, development of a rapid and sensitive technique for early detection of *Cronobacter* spp. in foods is of utmost importance.

To reduce or prevent the hazards posed by *Cronobacter* spp., a great deal of effort has been invested in the development of methods to eliminate *Cronobacter* spp. infection. The traditional biochemical procedures recommended by the US Food and Drug Administration (**FDA**) are laborious and time-consuming (FDA, 2002). To date, a variety of molecular detection and diagnostic techniques for *Cronobacter* spp. have been developed. The use of molecular methods, such as PCR, real-time PCR, loop-mediated isothermal amplification, and PCR-ELISA, has facilitated pathogen detection (Park et al., 2012; Cai et al., 2013; Shukla et al., 2016). Both PCR and real-time PCR assays have been increasingly exploited and characterized by a wide dynamic range of quantification, high sensitivity, and a high degree of precision (Hu et al., 2016; Li et al., 2016; Chen et al., 2017). Despite these improvements, these molecular methods are very complicated in terms of sample pretreatment and measuring procedures, are time-consuming, and require expensive laboratory apparatus and experienced technicians (Qiming et al.,

Received October 3, 2017.
Accepted January 16, 2018.
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2015; Zhou et al., 2016; Yu et al., 2017). Recently, the recombinase polymerase amplification (**RPA**) assay, an isothermal gene amplification method, has been developed as a potential strategy for various pathogens detection (Lillis et al., 2016; Wang et al., 2017a,b). The RPA assay not only is sensitive and specific but also has the advantages of rapidity and simplicity (Lillis et al., 2016; Abd El Wahed et al., 2015a,b).

In the present study, we developed RPA and real-time RPA for the detection of *Cronobacter* spp. in PIF for the first time by targeting the *ompA* gene. Our newly developed RPA and real-time RPA assays could be successfully used for sensitive and specific detection of *Cronobacter* spp. in contaminated PIF samples with a significantly reduced time consumption. The analysis time was reduced to less than 15 min, and our assays were as reliable as real-time PCR and the standard method GB4789.40–2016 (CFDA, 2016). Therefore, the RPA and real-time RPA assays serve as fast, reliable, and sensitive techniques for the detection of *Cronobacter* spp.

MATERIALS AND METHODS

Bacterial Stains

A total of 47 common pathogenic bacteria associated with dairy products and human health are shown in Table 1. The sources of these strains are listed. All strains were cultured in tryptic soy broth at 37°C.

Generation of Standard DNA

Bacterial genomic DNA was isolated using a TIANamp Bacterial DNA kit (Tiangen, Beijing, China) according to the manufacturer's instruction (http://www.tiangen.com/asset/imsupload/up0994144001433140765.pdf). The concentration of bacterial genomic DNA was quantified using a ND-2000c spectrophotometer (NanoDrop, Wilmington, DE). The copy number of DNA molecules was calculated by the formula amount (copies/μL) = [DNA concentration (g/μL)/(bacterial genome length in base pairs × 660)] × 6.02×10^{23}.

Primers and Exo Probe

Nucleotide sequences of *Cronobacter* spp. were aligned to identify conserved regions of the *ompA* gene. Primers and exo probes were designed based on the conserved region of the *ompA* gene using the Primer 5.0 software (Premier Biosoft International, Palo Alto, CA), as described in Table 2. The real-time RPA primers and probes were selected by testing the combination to yield the highest sensitivity. Primers and exo probes were synthesized by a commercial company (Sangon Biotech, Shanghai, China).

RPA and Real-Time RPA Assay

The RPA and real-time RPA reactions were performed in a 50 μL volume using a TwistAmp basic kit (TwistDX, Cambridge, UK) and TwistAmp exo kit (TwistDX, Cambridge, UK), respectively. Other components included 420 n*M* aliquots of each RPA primer, 14 m*M* magnesium acetate, and 1 μL of bacterial DNA. For the real-time RPA assay, 120 n*M* exo probe was also added to the mixture. The reaction tubes were placed in a Genie III scanner device (OptiGene Limited, West Sussex, UK) to start the reaction at 38°C. The fluorescence signal was recorded in real-time and increased markedly upon successful amplification.

Real-Time PCR

Real-time PCR was performed using ABI 7500 (Applied Biosystems, Foster City, CA) instrument. The sequences of the primers and probe are listed in Table 2. The Premix Ex Taq (Takara Co. Ltd., Dalian, China) was applied for real-time PCR, and the reaction was performed at 95°C for 30 s, followed by 35 cycles of 94°C for 5 s and 60°C for 34 s.

Analytical Specificity and Sensitivity Analysis

The analytical specificity was evaluated against a panel of pathogenic bacteria considered to be important in food (Table 1). To analyze the RPA sensitivity, genomic DNA from *Cronobacter sakazakii* (ATCC29544) was 10-fold serially diluted to achieve DNA concentrations ranging from 10^7 to 10^0 copies/μL. One microliter of each DNA dilution was used as a template and amplified with the RPA or real-time RPA assay. Real-time RPA was tested using the standard DNA in 8 replicates. The threshold time was plotted against the molecules detected.

PIF Spiking with Cronobacter spp.

Commercially available PIF was used to certify the potential use and the suitability of RPA assay. The PIF products were obtained from supermarkets in China. The absence of *Cronobacter* spp. was tested in PIF using the National Food Safety Standard of *Enterobacter sakazakii* Examination (GB4748.40–2016) of China (CFDA, 2016). One hundred grams of PIF and 10, 50, and 100 cfu of *Cronobacter* spp. were added to peptone water and then incubated for 6 or 8 h at 37°C. One milliliter of each bacterial culture was centrifuged