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 powdered 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.,

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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 $\text{amount (copies}/\mu\text{L}) = [\text{DNA concentration (g}/\mu\text{L}) / (\text{bacterial genome length in base pairs} \times 660)] \times 6.02 \times 10^{23}$.

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), respectively. Other components included 420 nM aliquots of each RPA primer, 14 mM magnesium acetate, and 1 μL of bacterial DNA. For the real-time RPA assay, 120 nM 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

Table 1. Bacterial strains and analytical specificity results for recombinase polymerase amplification (RPA) and real-time RPA assays

Strain name	Origin ¹	RPA ²	Real-time RPA ²
<i>Cronobacter sakazakii</i>	ATCC 29544	+	+
<i>Cronobacter sakazakii</i>	ATCC 29004	+	+
<i>Cronobacter sakazakii</i>	ATCC 12868	+	+
<i>Cronobacter sakazakii</i>	CICC 21560	+	+
<i>Cronobacter muytjensii</i>	ATCC 51329	+	+
<i>Cronobacter muytjensii</i>	CICC 21563	+	+
<i>Cronobacter malonaticus</i>	CICC 21551	+	+
<i>Cronobacter turicensis</i>	CICC 24178	+	+
<i>Cronobacter dublinensis</i>	CICC 21564	+	+
<i>Cronobacter universalis</i>	CICC 21570	+	+
<i>Campylobacter jejuni</i>	ATCC 33291	–	–
<i>Citrobacter freundii</i>	ATCC 10787	–	–
<i>Enterococcus faecalis</i>	ATCC 29212	–	–
<i>Listeria monocytogenes</i>	ATCC 19114	–	–
<i>Klebsiella pneumoniae</i>	ATCC 4352	–	–
<i>Proteus mirabilis</i>	ATCC 29906	–	–
<i>Providencia</i>	ATCC 29944	–	–
<i>Serratia marcescens</i>	ATCC 14756	–	–
<i>Staphylococcus aureus</i>	ATCC 6538	–	–
<i>Bacillus cereus</i>	CICC 10648	–	–
<i>Escherichia coli</i> O157:H7	CICC 21530	–	–
<i>Salmonella</i> Typhimurium	CICC 22956	–	–
<i>Shigella flexneri</i>	CICC 21678	–	–
<i>Shigella sonnei</i>	CICC 21679	–	–
<i>Vibrio parahaemolyticus</i>	CICC 21617	–	–
<i>Yersinia enterocolitica</i>	CICC 21609	–	–
<i>Bacillus cereus</i>	CMCC 63301	–	–
<i>Klebsiella pneumoniae</i>	CMCC 46117	–	–
<i>Proteus mirabilis</i>	CMCC 49005	–	–
<i>Salmonella</i> Paratyphi	CMCC 50671	–	–
<i>Salmonella</i> Paratyphi	CMCC 50094	–	–
<i>Salmonella</i> spp.	CMCC 50760	–	–
<i>Salmonella</i> spp.	CMCC 50540	–	–
<i>Shigella</i> spp.	CMCC 51197	–	–
<i>Shigella</i> spp.	CMCC 51062	–	–
<i>Shigella</i> spp.	CMCC 51334	–	–
<i>Staphylococcus aureus</i>	CMCC 26003	–	–
<i>Staphylococcus epidermidis</i>	CMCC 26069	–	–
<i>Staphylococcus albus</i>	CMCC 26101	–	–
<i>Staphylococcus pyogenes</i>	CMCC 26103	–	–
<i>Staphylococcus citreus</i>	CMCC 26106	–	–
<i>Streptococcus haemolyticus</i>	CMCC 3220	–	–
<i>Streptococcus haemolyticus</i>	CMCC 32210	–	–
<i>Bacillus licheniformis</i>	Isolated by our laboratory	–	–
<i>Enterobacter cloacae</i>	Isolated by our laboratory	–	–
<i>Mannheimia haemolytica</i>	Isolated by our laboratory	–	–
<i>Pseudomonas aeruginosa</i>	Isolated by our laboratory	–	–

¹ATCC = American Type Culture Collection; CICC = China Center of Industrial Culture Collection; CMCC = China Center for Medical Culture Collection; NCTC = National Collection of Type Cultures.

²+ = positive results; – = negative results.

Table 2. The sequences of primers and probes used in this study

Gene	Primer and probe
<i>ompA</i> (recombinase polymerase amplification)	Forward: 5' –GTAGACTTTACCTCGCCAGGGTGCTCATCAATA– 3' Reverse: 5' –CCGTCGTTAGGGATAAAGCCGGTATCGTGGAAC–3' exo probe: 5'–GCTATACCCCGGTGAAGGATTTAACCCTGAAC– (FAM-dT)-THF-(BHQ1-dT)-TCCCAAGGAAAAGCGCA-C3' spacer
<i>ompA</i> (real-time)	Forward: 5' –CATGGTATGGCGTGCTGACT– 3'' Reverse: 5' –CAGCGAATACCGGAGAAAACG–3' Probe: JOE-CTCTTCTAACATCGGCGGCGACGA-Eclipse

at $10,000 \times g$ for 3 min at 4°C. The supernatant was then discarded carefully and the cell pellet was washed with PBS. After centrifugation, the cell pellet was re-suspended and subjected to template extraction using a Bacterial Genomic DNA Extraction Kit (Tiangen). The bacterial genomic DNA samples were stored at −20°C until use.

RESULTS

Outer membrane protein A, which is essential for basolateral invasion of *Cronobacter* spp., was reported to be suitable for the detection of *Cronobacter* spp. (Cai et al., 2013). The *ompA* gene seems to have higher specificity than other genes, such as *ITS* (16S–23S rRNA internal transcribed spacer), 16S rRNA, *zpx*, and *gluA* and *gluB* (Jaradat et al., 2009; Cai et al., 2013). Based on the published DNA sequences of the *OmpA* gene of *Cronobacter* spp. and the results of BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) homology analysis, primers were designed using the Primer 5.0 software as described in Table 2. The primers amplified specific and significant band at 294 bp, as expected.

Analytical Specificity and Sensitivity of the RPA Assay

The analytical specificity of RPA assay was examined by using different pathogen genomic DNA as templates. As shown in Table 1, only *Cronobacter* spp. was detected successfully, including *Cronobacter sakazakii*, *Cronobacter muytjensii*, *Cronobacter malonaticus*, *Cronobacter turicensis*, *Cronobacter dublinensis*, and *Cronobacter universalis*, whereas other bacterial species were not detected. The results demonstrated the high specificity of the RPA assay.

The analytical sensitivity of RPA assay was determined by diluting the DNA concentrations of *Cronobacter* spp. from 10^7 to 10^0 copies/reaction. The specific 294-bp amplicon could be detected when the DNA concentration reached 10^4 copies/reaction (Figure 1A). To prove that RPA is a more rapid, technically simpler detection method, we examined the reaction time of the RPA assay. As shown in Figure 1B, positive results were obtained at 38°C within 10 min. The intensity of the 294-bp amplicon increased when the reaction time was up to 20 min; however, the intensity did not further increase after 20 min. These results indicated that the RPA assay was much faster than real-time PCR, which normally takes over 1 h.

Recently, real-time PCR assays have been exploited and characterized by high sensitivity. Therefore, we compared the analytical sensitivity of the RPA assay with that of real-time PCR. Figure 2A shows that

real-time PCR also had the same detection limit, 10^4 copies/reaction, using template DNA prepared from 10-fold serial dilutions of DNA of the same *Cronobacter* strain. The probit regression analysis on data from 8 runs demonstrated that the limit of detection (LOD) of real-time PCR was about 4.5×10^4 copies/reaction in 95% of cases (Figure 2B). These results indicated that the RPA assay possessed excellent and comparable analytical sensitivity to real-time PCR.

Analytical Specificity and Sensitivity of the Real-Time RPA Assay

To further improve the advantage of RPA assay, we developed real-time RPA by designing the specific exo probe based on the RPA assay. We first examined the analytical specificity of the real-time RPA assay. As shown in Table 1, real-time RPA detected only *Cronobacter* spp., but not other bacterial species. The results indicated the excellent specificity of the real-time RPA assay. The analytical sensitivity of the real-time RPA assay was determined by using the same diluted DNA concentrations of *Cronobacter* spp. as shown in Figure 1. The detection limit of real-time RPA was 10^4 copies/reaction, the same as those of real-time PCR and the RPA assay (Figure 3A). These results indicated that real-time RPA possessed prominent merits of analytical sensitivity and specificity, similar to real-time PCR.

With the average reaction time from 8 runs on the DNA standards, we made a semi-log regression analysis for the real-time RPA by using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA). The run-times of the real-time RPA assay were approximately 8 to 15 min for 10^7 to 10^4 copies (Figure 3B), whereas real-time PCR with cycle threshold values between 21 and 31 required approximately 32 to 45 min to obtain the final results. Moreover, a probit regression analysis using results of 8 complete molecular standard runs calculated that the LOD of real-time RPA was about 2.3×10^4 copies/reaction in 95% of cases (Figure 3C). These results indicated that the real-time RPA assay was much faster than but still as reliable as real-time PCR.

Validation of Real-Time RPA with Artificially Contaminated PIF

To accurately demonstrate the analytical sensitivity and high-speed of RPA and real-time RPA for detecting *Cronobacter* spp., PIF was contaminated artificially with different amounts of *Cronobacter* spp. for different enrichment times (Table 3). During enrichment procedures, the growth of other pathogens was considered an inhibitory factor for the target microbial flora, leading

to an increase in the detection limit. The diagnostic performance of the real-time RPA assay to detect *Cronobacter* spp. was compared with that of the traditional method (GB4789.40-2016) and real-time PCR. The traditional method is the national food safety standard method for *Cronobacter* spp. examination in China. This standard stipulates the testing method of *Cronobacter* spp. in food and applies to the examination of *Cronobacter* spp. in infant formula food, dairy products, and their raw materials. The traditional method is equivalent to the procedures recommended by the US FDA and ISO (FDA, 2002; ISO, 2006). After enrichment for 8 h, real-time RPA obtained positive results within 12.85, 10.8, or 8.18 min when PIF samples were contaminated with 10, 50, or 100 cfu/100 g of *Cronobacter* spp., respectively; whereas real-time PCR with cycle threshold values at 33.68, 33.12, and 26.93 required approximately 51, 50, and 41 min, respectively (Table 3). Using the traditional method, similar positive final results could be obtained when

contaminated samples were enriched for 8 h (Table 3). These results demonstrated that real-time RPA had a diagnostic agreement of 100% with real-time PCR and the traditional method. However, real-time RPA was much faster than real-time PCR and the traditional method. After 6 h of enrichment, none of these 4 methods could detect *Cronobacter* spp., indicating no false-positive results from samples containing a low level of *Cronobacter* spp. (Table 3). Therefore, the real-time RPA method is suitable for a reliable detection of low numbers of *Cronobacter* spp. in connection with an appropriate enrichment time.

DISCUSSION

Cronobacter spp. has become increasingly recognized as an opportunistic pathogen associated with neonatal infections resulting from the consumption of contaminated PIF (Kandhai et al., 2004). Although rare, infections caused by *Cronobacter* spp. are often life-

Figure 1. Analytical sensitivity and reaction time of recombinase polymerase amplification (RPA) assay. (A) The analytical sensitivity of RPA assay was determined by diluting the DNA concentrations of *Cronobacter* spp. from 10^7 to 10^0 copies/reaction. The specific 294-bp amplicon could be detected when the DNA concentration reached 10^4 copies/reaction. (B) Positive results were obtained at 38°C within 10 min. The intensity of 294-bp amplicon increased when the reaction time was up to 20 min.

threatening and most frequently cause meningitis, sepsis, and necrotizing enterocolitis (Willis and Robinson, 1988; Hunter and Bean, 2013). Because even low levels of *Cronobacter* spp. contamination could pose a health risk, accurate and sensitive methods are required for the rapid detection of *Cronobacter* spp. in food (Muytjens et al., 1988; Drudy et al., 2006; Pina-Pérez et al., 2016).

Here, we developed RPA and real-time RPA assays to rapidly identify the *Cronobacter* spp. with high analytical sensitivity and specificity. We examined the high specificity of RPA and real-time RPA with DNA from 47 bacterial strains. Among them, only *Cronobacter* spp. strains were correctly amplified, whereas no positive signal was observed from 37 nontarget pathogens.

Figure 2. Analytical sensitivity of real-time PCR assay. (A) Real-time PCR had the same detection limit, 10^4 copies/reaction. (B) Probit regression analysis based on the results of 8 runs using SPSS Statistics 22 software (IBM Corp., Armonk, NY). The limit of detection of 95% probability (4.5×10^4 copies) of real-time PCR is indicated by a rhomboid. Color version available online.

Table 3. Comparison of reaction time of different methods for *Cronobacter* spp. detection in contaminated powdered infant formula milk (PIF)¹

Spiked cells (cfu/25 g PIF)	Enrichment time (h)	Real-time RPA (min)	Real-time PCR (Ct)	RPA (min)	GB 4789.40	Viable cell counts (cfu/g)
10	6	ND ²	ND	ND	No	0
	8	12.51	33.68	20	Yes	3.5×10^2
50	6	ND	ND	ND	No	0
	8	10.48	33.21	20	Yes	2.0×10^3
100	6	ND	ND	ND	No	0
	8	8.11	26.93	20	Yes	2.4×10^3

¹RPA = recombinase polymerase amplification; GB4789.40 = Chinese traditional detection method (CFDA, 2016).

²ND = not detected.

Using the *Cronobacter* spp. plasmid DNA as the template, it was detected between 8 and 15 min for 10⁷ to 10⁴ copies of DNA. The LOD of real-time RPA was approximately 2.3 × 10⁴ copies/reaction, which was quite similar to that of PRA and real-time PCR using the same diluted *Cronobacter* spp. DNA in our study.

Figure 3. Performance of real-time recombinase polymerase amplification (RPA) for detection of *Cronobacter* spp. (A) Fluorescence development over time using a dilution range of 10⁷ to 10⁰ copies of the standard DNA. (B) Semilogarithmic regression of the data collected from 8 runs on the standard DNA using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA). The runtimes of real-time RPA were approximately 8 to 15 min for 10⁷ to 10⁴ copies. (C) A probit regression analysis. The limit of detection of the real-time RPA was approximately 2.3 × 10⁴ copies/reaction in 95% of cases and indicated by a rhomboid. Color version available online.

The detection rate of real-time RPA assay was comparable to that of real-time PCR, although the former assay we developed was much faster than the latter. In addition, the potential use of real-time RPA to detect *Cronobacter* spp. was demonstrated with artificially contaminated PIF samples. Our results showed that real-time RPA had a diagnostic agreement of 100% with real-time PCR and the traditional method.

Real-time RPA is a new pathogen detection technique with advantages over the traditional detection method and real-time PCR (Lillis et al., 2016; Wang et al., 2017a,b). Currently, RPA has been explored widely for molecular detection of diverse pathogens, and real field testing has also been achieved (Abd El Wahed et al., 2015a,b; Lillis et al., 2016). Furthermore, the portable point-of-care tube scanner (Genie III, OptiGene Limited) used in our study was much smaller, lighter, and cheaper than the real-time PCR machine and can be used in the field when run on battery power. Compared with DNA amplification methods, RPA assay has several advantages: (1) RPA does not require initial heating for DNA denaturation, which shortens the reaction time; (2) RPA shows a certain degree of tolerance to common PCR inhibitors; (3) RPA can tolerate 5 to 9 mismatches in the primer and probe without affecting the performance of the assay; and (4) RPA uses reagents stored in lyophilized pellets, which are stable and can react satisfactorily at 25°C for up to 12 wk and at 45°C for up to 3 wk. These distinguishing features of RPA and hands-on experience of real field testing using RPA make us believe that RPA could work excellently in a clinical diagnostic setting; real-time RPA serves as an efficient tool for implementation in food for the detection of *Cronobacter* spp. in less-advanced laboratories and is suitable for field detection.

CONCLUSIONS

In the present study, our newly developed RPA and real-time RPA assays could be successfully used for sensitive and specific detection of *Cronobacter* spp. in contaminated PIF with significantly reduced time consumption. The analysis time was reduced to less than 15 min, and the assays were as reliable as real-time PCR and the traditional method. All tests proved that the RPA and real-time RPA assays hold great promise for rapid, reliable, and sensitive detection of *Cronobacter* spp. in foods.

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