Dual-signal amplification strategy: Universal asymmetric tailing-PCR triggered rolling circle amplification assay for fluorescent detection of *Cronobacter* spp. in milk

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ABSTRACT

Cronobacter spp. are important opportunistic food-borne pathogens in powdered infant formula that cause many serious diseases in neonates and infants. In this study, a novel assay based on dual signal amplification strategy was developed by coupling asymmetric tailing PCR (AT-PCR) with rolling circle amplification (RCA) for the detection of *Cronobacter* spp. in milk. The tailing single-stranded DNA was generated through AT-PCR and used to initiate RCA, generating tandem repetitive G-quadruplex sequences. In the presence of the fluorescence dye thioflavin T that could intercalate into the G-quadruplex structures, the fluorescence signal was detected with a microplate reader. The AT-PCR coupled with RCA assay was specific for *Cronobacter* spp. detection because of the highly specific primers chosen for the AT-PCR. The limits of detection were 4.3×10^1 cfu/mL in pure culture and 4.5×10^2 cfu/mL in spiked milk, respectively. The fixed sequences designed in the hairpin DNA allowed this AT-PCR coupled with RCA assay to serve as a universal platform for the detection of other pathogens by modifying the specificity of the PCR primers.

Key words: *Cronobacter* spp., rolling circle amplification, asymmetric tailing PCR, fluorescence detection

INTRODUCTION

Cronobacter spp. are important opportunistic food-borne pathogens in powdered infant formula (PIF). The genus was previously described as yellow pigmented *Enterobacter cloacae* because of its ability to produce a yellow pigment (Urmenyi and White Franklin, 1961). In 1980, these organisms were designated as a new species,

Enterobacter sakazakii (Farmer et al., 1980). Then, it was reclassified as *Cronobacter* spp. This genus consists of 7 species, including *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter turicensis*, *Cronobacter muytjensii*, *Cronobacter dublinensis*, *Cronobacter universalis*, and *Cronobacter condimenti* (Iversen et al., 2007). According to the multi-locus sequence typing database (<https://pubmlst.org/cronobacter/>) reports (September 15, 2019), most isolates of *Cronobacter* spp. belonged to *C. sakazakii* (66.92%) and *C. malonaticus* (11.34%), indicating the importance of the 2 species in genus *Cronobacter*. Although *C. sakazakii* and *C. malonaticus* were mostly isolated from the environment or cases with the most severe neonatal prognosis (Ogrodzki and Forsythe, 2015), all species of *Cronobacter* spp. were considered to cause human illness except for *C. condimenti* (Cruz et al., 2011; Cruz-Córdova et al., 2012). *Cronobacter* spp. could cause necrotizing enterocolitis, meningitis, bacteremia, and sepsis with a 40 to 80% fatality rate in neonates (<28 d) and infants (Hunter et al., 2008). Many studies have identified PIF as the predominant source of neonatal infection by *Cronobacter* spp. and an increasing number of cases associated with PIF has been reported (Fei et al., 2015; Pei et al., 2016). The International Commission of Food Microbiology Specifications has identified *Cronobacter* as a severe hazard for restricted populations that could lead to life-threatening or substantial chronic sequelae or long duration (Skovgaard, 2003). The state administration for market regulation of China had proposed that *Cronobacter* spp. should not be present in infant formula (Chinese National Standard, 2010).

The development of an efficient and reliable method for the detection and assessment of *Cronobacter* spp. in PIF is of utmost importance. Traditional culture-based methods for *Cronobacter* spp. detection need pretreatment and selective enrichment, which are laborious (Pan et al., 2018; Song et al., 2018). To circumvent these disadvantages, many novel methods based on molecular biology techniques have been developed to

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achieve sensitive and rapid detection of *Cronobacter* spp. or other foodborne pathogens, such as PCR (Chen et al., 2017; Xu et al., 2017; Liang et al., 2019), loop-mediated isothermal amplification (**LAMP**; Fan et al., 2012; Hu et al., 2018a), and strand displacement amplification (Tang et al., 2017). In the past few years, PCR has been extensively used to detect pathogens because of its simplicity. Compared with conventional PCR, quantitative PCR could provide quantitative data but requires complicated instruments and experienced operators. A more convenient, specific, and sensitive method for the accurate detection and assessment of *Cronobacter* spp. in dairy products has been needed.

Asymmetric PCR (**aPCR**) is a kind of PCR method that could produce single-stranded DNA (**ssDNA**) by using unequal primer concentrations. In the first 10 to 15 cycles of the PCR process, the concentration of the 2 primers (limiting primer and excess primer) is sufficient, and a symmetric PCR process occurs. When the limiting primers are consumed (>15 cycles), the excess primers begin to dominate the linear amplification process, producing a large amount of ssDNA. The ssDNA amplicons produced via aPCR could be detected by agarose gel electrophoresis or used for further amplification. Moreover, although most signal amplification methods based on nucleic acid were reported and showed good sensitivity and simplicity, the application of nucleic acid signal amplification method to detect foodborne pathogens was limited because it is difficult to obtain short ssDNA from bacterial genomic double-stranded DNA. Compared with other nucleic acid-based methods that are usually designed for the detection of single-stranded DNA/RNA, the aPCR assay could provide an alternative and relatively convenient method for bacterial genomic detection (Low et al., 2013; Liu et al., 2015; Tian et al., 2018; Wang et al., 2018). The presence of ssDNA amplicons could lead to higher hybridization efficiency and detection sensitivity because of the absence of a competitive combination of complementary double strands (Wooddell and Burgess, 1996). The aPCR technique has potential for the detection of foodborne pathogens through its combination with other nucleic acid amplification or detection methods.

Rolling circle amplification (**RCA**) has been combined with fluorescence reagents to achieve fluorescent signal amplification (Huang et al., 2017; Zhang et al., 2019). Rolling circle amplification has been described as an isothermal enzyme-mediated amplification method for nucleic acid detection with higher sensitivity and simplicity (Liu et al., 2014). In a typical RCA process, DNA polymerase (e.g., Phi29 DNA polymerase or Bst DNA polymerase) replicates the circular template multiple times and generates a long ssDNA molecule with

repetitive sequences that are complementary to the circular DNA template (Zhao et al., 2008). The RCA process could be monitored or identified by using molecular beacons (Smolina et al., 2004), fluorescent dye (Qin et al., 2018), and other transduction molecules. Before the RCA process, the RCA template could be formed after the ligation of padlock DNA or dumbbell DNA in the presence of T4 DNA ligase (Thomas et al., 1999; Qing et al., 2017) and the ligation could also be designed as a recognition portion of the whole assay to achieve specific circularization or target detection. Moreover, RCA template could be designed to form a functional DNA structure such as a G-quadruplex to achieve signal output with the help of some specific reagents (Deng et al., 2017; Huang et al., 2019; Yang et al., 2019), making it a powerful and convenient method for nucleic acid detection.

In this study, a sensor based on dual-signal amplification strategy has been developed using a combination of asymmetric tailing PCR (**AT-PCR**) with RCA, AT-PCR + RCA. A circular DNA template with complementary G-quadruplex DNA sequence could be obtained via the ligation of pre-designed hairpin DNA. The tailed ssDNA amplicons that were obtained from AT-PCR could initiate the RCA process to form G-quadruplex DNA structures. With the addition of G-quadruplex-specific fluorescent dye thioflavin T (**ThT**), which intercalated with the G-quadruplex DNA structures, fluorescence signals could be detected with a microplate reader. Owing to the fixed initial sequence in the RCA template, the strategy was applicable to detect other pathogens or ssDNA without altering the hairpin DNA sequence, making it a low-cost and universal platform for pathogen detection. The AT-PCR + RCA sensor showed good detection sensitivity, specificity, and universality with great potential application in pathogen detection.

MATERIALS AND METHODS

Materials and Reagents

Primers and hairpin DNA used in this experiment were purchased from Tsingke Biological Co. Ltd. (Beijing, China). The DNA sequences were listed in Table 1. The relevant characterization information of hairpin DNA was shown in Supplemental Figure S1 (<https://doi.org/10.3168/jds.2019-17590>). The sequence of hairpin DNA was validated by The Nucleic Acid Package analysis software (<http://www.nupack.org>) to guarantee the absence of undesired mismatch and structures (Supplemental Figure S2; <https://doi.org/10.3168/jds.2019-17590>). The sequences of *ompA* (outer membrane protein, *ompA*) gene (GenBank: GQ845410.1)

Table 1. Oligonucleotide sequences used in this assay¹

DNA	Sequence (5'–3')	Amplicon size (bp)	Reference
Hairpin DNA	Phosphate-CAGAACAGGTTTAAAAAAAAAAAAAAAAAAAAAACCACCC GCCCTACCCCTTTCCTGTTCTG	NA	This study
<i>OmpA</i> -F	GAGAAACGTGCTCAGTCTGT	109	This study
<i>OmpA</i> -R	AAAAAAAAAAAAAAAAAAAAATGGATTGCGCCATACCACGTGC	NA	This study
P1	TTTTTTTTTTTTTTTTTTTTTTT		

¹F = forward; R = reverse; NA = not available. Underlined sequences represent complementary region of P1.

of *Cronobacter* spp. were downloaded from National Center for Biotechnology Information database (<http://www.ncbi.nlm.nih.gov/>). The specific primers targeting *ompA* gene of *Cronobacter* spp. for AT-PCR assay were selected based on multiple sequences alignment analysis (Supplemental Figure S3; <https://doi.org/10.3168/jds.2019-17590>) using ClustalW algorithm in MEGAX (Kumar et al., 2018) and designed following the rules of linear-after-the-exponential PCR principles described by Pierce et al. (2005) to maximize AT-PCR efficiency. The nucleotides and oligonucleotides were dissolved in sterilized ultrapure water (18.3 Ω cm⁻¹, Millipore, Bedford, MA) and stored at -20°C. The T4 DNA ligase (5 U/ μ L), Phi29 DNA polymerase (10 U/ μ L), DL500 DNA marker, DL5000 DNA marker, and dNTP mixture (2.5 mM) were purchased from Sangon Biotech Co. Ltd. (Shanghai, China). Two $\times$ Taq Master Mix (Dye) for PCR was purchased from Novoprotein Technology Co. Ltd. (Shanghai, China). Bovine serum albumin and ThT were purchased from Sigma-Aldrich (St. Louis, MO). The agarose was obtained from Solai Bao Technology Co. Ltd. (Beijing, China). All reagents used were of analytical grade.

Bacterial Strain Information

The target strains belonging to *Cronobacter* spp. were *C. sakazakii* ATCC29544, *C. muytjensii* ATCC51329, *C. turicensis* CICC24178, *C. condimenti* CICC24184, *C. dublinensis* ssp. *dublinensis* CICC24179, *C. malonaticus* CMCC45402, and *C. universalis* NCTC9529. Nontarget strains, including *Staphylococcus aureus* CMCC26001, *Pseudomonas aeruginosa* CMCC10104, *Listeria monocytogenes* ATCC13932, *Listeria monocytogenes* CMCC54007, *Listeria seeligeri* ATCC35967, *Salmonella* Typhi JX-CDC40002 (Jiang Xi Province Center for Disease Control and Prevention, Nanchang, China), *Salmonella* Enteritidis ATCC13076, *Salmonella* Typhimurium ATCC13311, *Escherichia coli* ATCC25722, *Escherichia coli* O157:H7 CMCC44828, *Escherichia coli* O157:H7 ATCC43888, *Bacillus cereus* ATCC14579, *Franconibacter pulveris* LMG24057, *Franconibacter helveticus* LMG23732, and *Siccibacter turicensis*

LMG23730 were used to validate the specificity of the assay. Strains were revived and grown in Luria-Bertani medium at 37°C in a rotary shaker at 180 rpm for overnight. Plate count assay using Luria-Bertani medium for the determination of bacterial concentrations was followed by serial dilutions with sterile PBS (0.01 M, pH 7.4) and incubation at 37°C, 12 h.

Sample Preparation

Genomic DNA of *C. sakazakii* ATCC29544 was extracted with the boiling method. One milliliter of overnight culture was centrifuged at 12,000 $\times g$ for 5 min at room temperature. The pellet was washed twice with PBS and resuspended with 50 μ L of sterilized ultrapure water and placed in a water bath for 15 min. After centrifugation at 12,000 $\times g$ for 5 min at room temperature, genomic DNA was obtained from the supernatant. Then AT-PCR was applied to generate and amplify the ssDNA from bacterial genomic DNA. A total of 50 μ L of the AT-PCR reaction contained 25 μ L of 2 $\times$ Taq Master Mix, 3 μ L of genomic DNA, 1 μ M forward *ompA* primer, 0.2 μ M reverse *ompA* primer, and then 16 μ L of sterilized ultrapure water was added. The AT-PCR process was initiated with 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, and final elongation at 72°C for 10 min.

Formation of the Dumbbell-Like DNA from Hairpin DNA

The RCA template, circular probe (dumbbell-like DNA) could be formed between 2 hairpin DNA molecules by the ligation of T4 DNA ligase. Before the ligation, the hairpin DNA was heated at 95°C for 5 min, then the mixture was cooled to room temperature, which allowed the formation of the desired hairpin structure. Ligation process was performed in a total of 100 μ L volume reaction that contained 10 μ L of hairpin DNA (1 μ M), 8 μ L of T4 DNA ligase, 10 μ L of 10 $\times$ T4 DNA ligase reaction buffer, and 72 μ L of sterilized ultrapure water. The reaction was carried in a thermal

cycler at 22°C for 2 h, 65°C for 10 min to inactivate the ligase and terminate the ligation. The dumbbell-like DNA was either directly used or stored at −20°C.

RCA Assay and Fluorescent Detection

The RCA assay for signal amplification was performed by using AT-PCR amplicons or synthetic poly-T oligonucleotide primer P1 (Table 1). A 50-μL volume of the RCA reaction contained 12.5 μL of dumbbell-like DNA, 10 μL of AT-PCR amplicons or primer P1, 1 μL of phi29 DNA polymerase (10 U/μL), 5 μL of 10 × phi29 DNA polymerase reaction buffer, 5 μL of dNTP (2.5 mM), 2.5 μL of BSA (100 μg/mL), 1 μL of ThT (1 mM), and 10.5 μL of sterilized ultrapure water. The reaction mixture was incubated at 30°C for 3 h and transferred to a microplate (black) for fluorescent detection. Fluorescence intensities were determined using a Varioskan multimode microplate reader (Thermo Fisher Scientific, Waltham, MA). The excitation wavelength was set at 425 nm and the emission wavelength was measured at a range between 450 to 600 nm.

Agarose Gel Electrophoresis Analysis

The formation of the dumbbell-like DNA and RCA products were analyzed in agarose gel (5% for dumbbell-like DNA and 1% for RCA products) electrophoresis with 1 × TAE buffer (0.04 M Tris–acetate, 2 mM EDTA, pH 8.5) at 70 V for 50 min at room temperature. The results were visualized and captured by ImageQuant LAS 500 (GE Healthcare Bio-Sciences, Pittsburgh, PA).

Limit of Detection and Specificity of the Proposed Strategy in Pure Culture

The limit of detection (LOD) of the method was tested with a 10-fold *C. sakazakii* ATCC29544 fresh pure culture dilution series ranging from 4.3×10^6 to 4.3×10^1 cfu/mL. Genomic DNA extraction, AT-PCR, and RCA with fluorescence detection were performed as described above. Moreover, other target strains belonging to *Cronobacter* spp. and nontarget strains were also tested to verify the specificity of the method.

Detection of *C. sakazakii* in Artificial Contaminated Milk

To validate the feasibility of the AT-PCR + RCA sensor in real milk matrix samples, 1 g of sterile skim milk powder was dissolved in 9 mL of PBS to obtain a 10% skim milk powder matrix (milk matrix). A total

of 50 μL of various concentrations of *C. sakazakii* fresh pure culture were added to every 450-μL milk matrix to get bacterial final concentrations ranging from 4.5×10^6 to 4.5×10^0 cfu/mL. After genomic extraction, the AT-PCR coupled with RCA assay was performed to assess the LOD in the milk matrix.

RESULTS AND DISCUSSION

Experimental Principle

The schematic of the proposed strategy for the detection of *Cronobacter* spp. in milk was illustrated in Figure 1. The 5'-end phosphorylated hairpin DNA was designed for RCA samples. Its sequence contained 3 parts: primer binding region (21-adenine), complementary sequences for the G-quadruplex, and complementary double-stranded stem sequences. The entire assay includes 3 parts: (a) ligation of the hairpin DNA to form the dumbbell-like DNA, (b) AT-PCR assay to obtain target ssDNA amplicons, and (c) the target ssDNA combined with dumbbell-like DNA to initiate the RCA process. (a) The dumbbell-like DNA was preformed using T4 DNA ligase to ligate the 2 hairpin DNA after which the T4 DNA ligase was inactivated to eliminate possible interference with phi29 DNA polymerase. (b) Genomic DNA of *C. sakazakii* obtained by boiling the samples in water bath was used for the AT-PCR. The specific primer targeted the *ompA* gene of *Cronobacter* spp., whereas the 5'-end of the reverse primer was tailed with 21-adenine. (c) After the AT-PCR amplification of the genomic DNA, the short ssDNA amplicons that were tailed with 21-thymine could partly bind to the primer binding region of the dumbbell-like DNA. This binding resulted in the initiation of the RCA process, which generated a long and repeated ssDNA that contained G-quadruplex sequences that resulted from the 21-adenine from the 5'-end of the reverse primer. In the presence of fluorescence dye ThT and K^+ , the repeated G-quadruplex structures could be formed and ThT intercalated into the G-quadruplex structures with high affinity that resulted in significant fluorescence enhancement that was excited at 425 nm and emitted at 490 nm (Mohanty et al., 2013).

Feasibility of the Proposed Strategy

Agarose gel electrophoresis with ethidium bromide stain and microplate fluorescence detection were carried out to confirm the results at the various stages in the development of the AT-PCR coupled with RCA assay. Initially, gel electrophoresis was used to follow the ligation process that formed the dumbbell-like

Figure 1. Schematic illustration of the asymmetric tailing PCR (AT-PCR) coupled with rolling circle amplification (RCA) assay for the detection of *Cronobacter* spp. ThT = fluorescence dye thioflavin T.

DNA. As shown in Figure 2, in the presence of T4 DNA ligase, a new distinct band of dumbbell-like DNA (lane 2) with slower mobility than the hairpin DNA (lane 1) was observed in 5% agarose gel, indicating the ligation between the 2 hairpin DNA to form the dumbbell-like DNA. After ligation, RCA was carried out with a synthetic primer P1 that resulted in a visible and distinct fluorescence signal that was obtained only in the presence of both dumbbell-like DNA and P1, as shown in Figure 3A, suggesting that P1 could trigger the RCA process and generated the G-quadruplex sequences. The ThT intercalation, which resulted in fluorescence signal, was used to confirm the formation of G-quadruplex. However, a mild fluorescence signal was observed when the reaction mixture contained dumbbell-like DNA, which could have resulted in the random and nonspecific amplification of phi29 DNA polymerase in the presence of dNTP.

A 1% gel electrophoresis was performed to confirm the initiation of the RCA process. As shown in Figure 3B, the RCA products (lane 3) exhibited a significant fluorescence signal, suggesting successful initiation of the RCA process. These results indicated that the hairpin DNA formed the dumbbell-like DNA and the

Figure 2. Agarose gel electrophoresis verification of the dumbbell-like DNA formation. Lane M: DL500 DNA Marker (Sangon Biotech Co. Ltd., Shanghai, China); lane 1: hairpin DNA (1 μ M); and lane 2: hairpin DNA (1 μ M) + T4 DNA ligase + T4 DNA ligase buffer.

Figure 3. Verification of the rolling circle amplification (RCA) process using primer P1. (A) Fluorescence verification of the RCA process using (a) dumbbell-like DNA with primer P1, (b) dumbbell-like DNA, and (c) primer P1. (B) Agarose gel electrophoresis verification of the RCA process. Lane M: DL5000 DNA Marker (Sangon Biotech Co. Ltd., Shanghai, China); lane 1: RCA system with primer P1; lane 2: RCA system with dumbbell-like DNA; and lane 3: RCA system with dumbbell-like DNA and primer P1. a.u. = arbitrary units.

RCA process was initiated successfully in the presence of primer P1.

Optimization of Conditions

To obtain optimized conditions for the AT-PCR + RCA sensor, the concentrations of the dumbbell-like DNA and ThT were optimized using P1 at 1 μM as the RCA initiator. As shown in Figure 4A, when the concentration of the dumbbell-like DNA was set from 200 to 350 nM, the fluorescence signal was increased and reached a plateau at 250 nM. So, the optimized concentration of dumbbell-like DNA in this assay was 250 nM. When the concentration of ThT was set from 2.5 to 30 μM , the fluorescence signal increased and finally reached a plateau at 20 μM . Hence, the optimized

concentration of ThT that was used in the succeeding assays was 20 μM (Figure 4B).

AT-PCR Efficiency Optimization

The primers for AT-PCR assay were designed following the principle described in by Pierce et al. to achieve relatively maximal aPCR efficiency. The melting temperature of limiting primer (70.1°C) is higher than that of excess primer (63.5°C), so the efficiency of the linear phase of AT-PCR can be improved. The detailed design principle can be found in this previous study (Pierce et al., 2005).

Since the ratio of forward primer and reverse primer was a critical parameter of the AT-PCR assay, it was optimized to improve the efficiency of the AT-PCR

Figure 4. Optimization of the concentrations of (A) dumbbell-like DNA and (B) fluorescence dye thioflavin T (ThT). The fluorescence intensities were measured at emission wavelength = 490 nm. a.u. = arbitrary units. Error bars indicate the SD from 3 representative experiments.

Figure 5. Optimization of the asymmetric tailing PCR (AT-PCR) forward/reverse (F/R) primer ratio. (A) Agarose gel electrophoresis verification. Lane M: DL500 DNA Marker (Sangon Biotech Co. Ltd., Shanghai, China); lane C: control; lane 1: primer ratio of F/R was 1:1; lane 2: primer ratio of F/R was 5:1; lane 3: primer ratio of F/R was 10:1; and lane 4: primer ratio of F/R was 20:1. (B) Fluorescence detection. The fluorescence intensities were measured at emission wavelength = 490 nm. a.u. = arbitrary units. Error bars indicate the SD from 3 representative experiments.

assay. As shown in Figure 5A, AT-PCR with various ratios of forward and reverse primers (F/R) were set at 1:1, 5:1, 10:1, and 20:1 to optimize the F/R. Using unequal F/R ratios generated bright bands of ssDNA at 5:1 and 10:1, which was consistent with our previous studies in the aPCR assay, but the bands presented in this AT-PCR assay were brighter (data not shown). To further evaluate the optimal F/R ratio, RCA was performed. In Figure 5B, when the ratio of F/R was 5:1, the fluorescence intensity was the highest, indicating this to be the best F/R ratio among the conditions tested. This also indicated that the AT-PCR products initiated the RCA process and generated G-quadruplex DNA sequences.

Performance Analysis of the Strategy

The sensitivity of the AT-PCR + RCA sensor was validated using the optimized conditions as described above. *C. sakazakii* ATCC29544 overnight pure culture (about 4.3×10^9 cfu/mL) was diluted into a 10-fold dilution series ranging from 4.3×10^6 to 4.3×10^1 cfu/mL. Genomic DNA extraction, aPCR assay, and RCA assay were followed, and fluorescence intensities were collected with a microplate reader. As shown in Figure 6A, the fluorescence intensity increased with increasing concentrations of *C. sakazakii*. A linear relationship between the fluorescence intensity and the concentration of *C. sakazakii* was achieved over a dynamic range from 4.3×10^6 to 4.3×10^1 cfu/mL. The linear regression equation (Figure 6B) in pure culture was given by $y = 4.151x - 1.187$ ($R^2 = 0.9562$), where y = the fluorescence intensity measured at 490 nm and x = the log concentration of *C. sakazakii*. Based on the cut-off

value (mean + 3 SD of the control group), the LOD of the proposed strategy in pure culture was 4.3×10^1 cfu/mL.

Specificity and Universality Evaluation

To determine the specificity of the proposed strategy, several common foodborne pathogens and species belonging to *Cronobacter* (with concentrations of 10^6 cfu/mL) were analyzed using the optimized parameters. As shown in Figure 7, the group containing *Cronobacter* spp. (Figure 7A) showed higher fluorescence intensities than control group, but the nontarget bacteria (Figure 7B) showed similar fluorescence intensities with the control group, indicating that the AT-PCR amplicons from the nontarget bacteria tested could not initiate the RCA process. These results indicated that the components of the AT-PCR + RCA sensor developed in this study were highly specific for the detection of *Cronobacter* spp. The specificity of this assay was resulting from the highly specific primers chosen for the AT-PCR. As a *Cronobacter* species-specific gene, the *ompA* gene has been widely used as a target for the detection of *Cronobacter* spp. in PIF with high specificity and sensitivity (Chen et al., 2017; Liu et al., 2018). These studies indicated that *ompA* gene exists in all the 7 species of *Cronobacter*. However, due to the absence of a suitable variable domain in the *ompA* gene, the AT-PCR primers designed in this assay are not applicable for the identification of *Cronobacter* species, making it a weakness of this method (Hu et al., 2018b).

Furthermore, to verify the universality of the AT-PCR + RCA sensor, some other foodborne pathogens including *Pseudomonas aeruginosa* CMCC10104,

Figure 6. (A) Fluorescent signal response with different concentrations of *Cronobacter sakazakii* in pure culture ranging from 4.3×10^6 to 4.3×10^1 cfu/mL. (B) The calibration curves of the log *C. sakazakii* concentrations (cfu/mL) with corresponding fluorescence intensities. The fluorescence intensities were measured at emission wavelength = 490 nm. The dashed line represents the cut-off value. Error bars indicates the SD from 3 representative experiments. a.u. = arbitrary units.

Escherichia coli O157:H7 ATCC43888, and *Salmonella* Enteritidis ATCC13076 were detected. Three pairs of primers (Supplemental Table S1; <https://doi.org/10.3168/jds.2019-17590>) were selected based on their specific genes, respectively, and modified with 21-adenine (forward or reverse primer); the position of the 21-adenine depended on their efficiency in AT-PCR (data not shown). Distinct fluorescent signals could be observed in all 3 pathogens compared with the control group, as shown on Supplemental Figure S4 (<https://doi.org/10.3168/jds.2019-17590>). This indicated that the AT-PCR + RCA sensor has the potential to be a universal

platform for the detection of foodborne pathogens with modifications to the primers.

Detection of *C. sakazakii* in Artificially Contaminated Milk Sample

The feasibility of the proposed strategy in spiked milk matrix was demonstrated using serial concentrations of *C. sakazakii*. As shown in Figure 8A, the fluorescent intensities increased with increasing *C. sakazakii* concentrations in the spiked milk matrix containing 4.5×10^6 to 4.5×10^0 cfu/mL. A linear relationship (Figure

Figure 7. Specificity verification with (A) target *Cronobacter* spp. strains and (B) nontarget strains. The fluorescence intensities were measured at emission wavelength = 490 nm. a.u. = arbitrary units. Error bars indicate the SD from 3 representative experiments.

Figure 8. (A) Fluorescent signal response with different concentrations of *Cronobacter sakazakii* in spiked milk matrix ranging from 4.5×10^6 to 4.5×10^0 cfu/mL. (B) The calibration curves of the log *C. sakazakii* concentrations (cfu/mL) with corresponding fluorescence intensities. The fluorescence intensities were measured at emission wavelength = 490 nm. The dashed line represents the cut-off value. Error bars indicate the SD from 3 representative experiments. a.u. = arbitrary units.

8B) between fluorescent intensities and the concentrations of *C. sakazakii* was given by $y = 4.247x - 4.391$ ($R^2 = 0.9660$), where y = the fluorescence intensity measured at 490 nm and x = the log concentration of *C. sakazakii*. Based on the cut-off value (mean + 3 SD of the control group), the LOD of the proposed strategy in spiked milk matrix was 4.5×10^2 cfu/mL. In our previous studies in foodborne pathogen detection (Yu et al., 2019; Zhan et al., 2019) or other *Cronobacter* detection methods (Supplemental Table S2; <https://doi.org/10.3168/jds.2019-17590>) in past or recent years, assays based on antibody, PCR, and isothermal amplification were applied to the detection of *Cronobacter*. In these studies, antibody-based assays have the advantage of being rapid and convenient, but the preparation of antibodies specific to all species of *Cronobacter* is quite difficult. On the other hand, although other isothermal amplification-based assays such as LAMP possessed the advantages of high sensitivity, complicated primer design and annoying false-positive signal hampered the application of this method. The proposed strategy presented in this study did not require the preparation of anti-*Cronobacter* antibodies or design of complicated LAMP primers, it showed a comparable sensitivity (10^2 cfu/mL) with high specificity, providing an alternative more convenient nucleic acid-based method for *Cronobacter* spp. detection in PIF.

CONCLUSIONS

A sensitive and highly specific AT-PCR + RCA universal sensor has been developed for the detection of *Cronobacter* spp. The sensor could detect as low as 4.3×10^1 cfu/mL *C. sakazakii* in pure culture and 4.5×10^2 cfu/mL in spiked milk matrix. With a modification

of the primers for AT-PCR, this sensor could serve as a universal platform for the detection of other pathogens. The strategy presents a simple and low-cost universal platform for sensitive and specific detection of food-borne pathogens especially in milk products.

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