Development of a simultaneous amplification and testing (SAT) system based fluorescence real-time isothermal RNA amplification for rapid detection of *Cronobacter* spp. in powdered infant formula

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Abstract

Contamination of *Cronobacter* spp. in powdered infant formulas is a severe food safety problem. The present study developed a rapid and sensitive Simultaneous Amplification and Testing (SAT) system for the detection of *Cronobacter* spp. in powdered infant formula. SAT detection system is based on fluorescence real-time isothermal RNA amplification and mainly includes pre-enrichment, RNA isolation and detection by fluorescence real-time RNA isothermal amplification. The amplification targets 16 s/23 s rRNA for the specific detection and rapid identification of *Cronobacter* spp. and can accurately detect viable strains in infant formulas and other food products. Here, six *C. sakazakii* strains and 25 references strains were examined using one pair of primers, having the accuracy of 100% in reference to conventional methods like ISO-IDF 22964 and real-time PCR. The SAT assay was proved to be highly sensitive with a detection limit of 10^2 CFU/mL without pre-enrichment for powdered infant formula. After 3 h, 4 h and 8 h enrichment, the sensitivity was increased up to 10^0, 10^{-1} and 10^{-3} CFU/mL of *Cronobacter* spp., respectively. The SAT system including pre-enrichment performed for *Cronobacter* spp. detection was less than 4 h, dramatically shortened, in comparison to several days using standard culturing method and overnight using pre-enrichment real-time PCR method. And more importantly, the SAT assay can accurately distinguish viable strains from the dead one. Taken together, the SAT assay combined with pre-enrichment established in the present study should provide a rapid, sensitive, efficient and specific method for direct detection of *Cronobacter* spp. in powdered infant formula. Moreover, a full automatic food-borne pathogenic bacteria detector was developed based on the SAT assay.

Introduction

Neonatal infections caused by *Cronobacter* spp. (formerly *Enterobacter sakazakii*) have been reported from different parts of the world. *Cronobacter* causes meningitis and necrotizing enterocolitis in infants, and septicemia and catheter-associated infections in immunocompromised people and elderly, with mortality rates ranging from 10 to 80% (Mullane et al. 2007). All *Cronobacter* spp. have been associated with human infections excepted *C. condiment* (Cruz-Córdova et al. 2012). *Cronobacter* spp. is a group of Gram-negative pathogenic bacteria belonging to the family Enterobacteriacea, which can survive in the intestine of humans and animals. The multi-species complex is typically facultative anaerobic, catalase-positive, oxidase-negative, motile, rod-shaped, non-spore forming bacteria that can produce a yellow pigment. Although *Cronobacter* can be rehabilitated through the use of antibiotics, the mortality rate of infection caused by the bacteria is up to 50%, accompanied by severe neurological sequelae, developmental disorders and other symptoms.

It has been reported that *Cronobacter* spp. are highly diverse and share many phenotypic traits with other *Enterobacteraceae* members (Jaradat et al. 2009). They are markedly resistant to osmotic stress and dryness and moderately thermotolerant (Jaradat et al. 2009). For example, some encapsulated *Cronobacter* spp. were still recoverable from desiccated infant formula after 2.5-year-storage (Barron and Forsythe 2007; Breeuwer et al. 2003). It is also known to survive for 2 years in the powdered infant formula (PIF) at low water activity (Edelson-Mammel et al. 2005). In addition, the growth of this group of

microorganisms has also been recorded in PIF reconstituted at temperatures ranging from 8 to 47 ℃ (Iversen et al. 2008). Therefore, the potential risk of *C. sakazakii* contamination during the preparation of PIF is considered high (O'Brien et al. 2009).

Cronobacter spp. contaminated PIFs was identified as the source of infection (Friedemann 2009). For PIF, current microbiological criteria require the absence of all seven *Cronobacter* species in 30 samples of 10 g (Codex Alimentarius Commission 2008; Jackson and Forsythe 2016). The losses caused by *Cronobacter* can be significant for manufacturers. For example, in 2011, a suspected outbreak of *C. sakazakii* in the United States led to product recalls and a subsequent 10% drop in the manufacturers' shares although the laboratory evidence to linking their product to infant infections and deaths was lack (Centers for Disease Control 2016; Geller 2011; Jackson and Forsythe 2016). Moreover, the costs of infection are also high because of the long-term influences of the disease like life-long brain damage. It was estimated that the cost of *C. sakazakii* infections was more than $5 million per case (Minor et al. 2015). Taken together, the losses and costs of *C. sakazakii* infections from PIFs are enormous.

A variety of biological techniques have been developed for detection of *Cronobacter spp*., including the traditional isolation and biochemical identification after enrichment, or differential screening medium. Culture dependent isolation and assay methods for complete analysis of *Cronobacter* spp. are time-consuming and laborious (International Organization for Standardization 2006a; Chen 2012). In addition, several molecular methods have been reported, including conventional PCR assay using 16 s rRNA (Lehner et al. 2004), *OmpA* or *rpoB* genes (Mohan Nair and Venkitanarayanan 2006; Stoop et al. 2009), real-time PCR using *OmpA* (Cai et al. 2013; Zimmermann et al. 2014), the fluorescence in situ hybridization (FISH) using a novel peptide nucleic acid probe (Almeida et al. 2009) and the loop-mediated isothermal amplification (LAMP) using 16S/23S rDNA (Liu et al. 2012). Although the molecular methods offer efficient approaches for the rapid detection of *Cronobacter*, they are still unfeasible for on-site inspection due to the requirement of professional equipment and expensive labelled probe and the costing of time. Moreover, LAMP may have high risk of amplicon contamination (Cai et al. 2013).

In order to rapidly and accurately identify viable *Cronobacter* species in PIF and its associated environment, in the present study, a real time fluorescent reverse transcription loop-mediated isothermal amplification system (RT-LAMP) targeting 16S/23S rRNA were developed using the simultaneous amplification and testing (SAT) technique. For SAT technique, RNA is used as a template to amplify at isothermal condition 42 ºC, and the amplification products are mixtures of RNA and DNA with high efficiency. It has the advantages of less test time (2 ~ 12 h), higher accuracy and no pollution, and has the ability to distinguish between dead and live bacteria.

Materials And Methods

Bacterial strains, Pre-Enrichment and media

The 6 reference strains of *Cronobacter* spp. used in this study were purchased from China Center of Industrial Culture Collection (CICC). Other 25 non-*Cronobacter* reference strains (Table 1) including

Citrobacter, Enterobacter, Escherichia, Klebsiella, Listeria, Proteus, Pseudomonas, Salmonella, Shigella, Vibrio and *Yersinia*, were obtained from CICC□American Type Culture Collection (ATCC) and National Center for Medical Culture Collections (CMCCB). All the stains were maintained on Luria-Bertani (LB) plates. In order to recover bacteria strains, aliquots of 1 mL of the enrichment broth were aseptically added to 9 mL of LB broth, and incubated at 37 ℃ for 8-12 h. After incubation, the amount of colonies was calculated.

Total RNA extraction

Single colony was picked up from agar plate and inoculated into 3 mL LB broth in a flask. Each bacteria was incubated at 37 ºC for 12 h with shaking at 150 rpm. Total RNA of all strains was isolated using Bacterial RNA Kit according to the manufacturer's instructions (Omega Bio-tek, Norcross, GA. USA). After treatment with RNase-free DNase I (Omega Bio-tek), RNA samples were measured using a Beckman DU800 (Beckman, Indianapolis, IN, USA) (Lv et al. 2019). Genomic DNA of all strains was extracted using Bacterial DNA Kit according to the manufacturer's instructions (Omega Bio-tek).

Development of a simultaneous amplification and testing (SAT) system

SAT reaction was performed in a total 40 μL mixture consisted of 1 μL of M-MuLV reverse (200 U/μL, Roche, Basel, Switzerland), 2 μL of T7 RNA Polymerase (200 U/μL), 4 mM $MgCl_2$, 0.4 mM dNTP/analog mixture for each, 0.5 mM of each NTP, 0.5 mM of primers for each (SAT-CF: 5'-TGACATCCAGAGAATCCTG-3' and SAT-CR: 5'- ACAACATTTCACAACACGAGCC-3'), 0.5 mM probe (5'-CACCGAGATGCGGGAGTGCGGTG-3'), and 1 μL of RNA template. Thermal cycling parameters were as follows: 60 ºC, 10 min; 42 ºC, 5 min; 60 cycles of amplification at 42 ºC for 50 s. And, the fluorescence was collected at the end of each cycle.

Real-Time PCR reaction conditions

Real-time PCR reaction was performed in a total 25 μL mixture containing 0.5 μL of Taq DNA polymerase, 4 mM $MgCl_2$, 0.4 mM of each dNTP, 0.5 mM of each NTP, 0.5 mM each primer (RT-CF: 5'-GGCGAGCGGCGAATATTAT-3' and RT-CR: 5'-CGGGTTTTCCCAGTTGAGATC-3'), 0.5 mM probe (5'-FAM-CACCAGTTTTCGGTGCGCCAGC-BHQ-3'), and 2 μL of DNA template. The PCR was carried out according to the follow parameters: a denaturation at 37 ℃ for 5 min, a denaturation at 95 ℃ for 3 min, then 40 cycles of denaturation at 95 ℃ for 5 s, annealing at 60 ℃ for 40 s.

Specificity of the SAT for C. sakazakii

To evaluate the specificity of SAT assay, 6 *C. sakazakii* strains and 25 reference strains were cultured in Luria-Bertani (LB) broth at 37 ℃ for 16-18 h to about 10^8 CFU/mL. All bacteria strains were counted on LB agar plate at 37 ℃ overnight. The ten-fold serial dilutions (10^7-10^0 CFU/mL) were obtained using saline solution. Total RNA of all bacterial strains was extracted as described above and used as template in the subsequent SAT test. The specificity of the results was based on the melting curve analysis and

real-time PCR amplification curve. Moreover, the sequence of the amplicons was further confirmed via sequencing (Sangon Biotech, Beijing, China) and was subsequently aligned with corresponding sequences available in Genbank.

To evaluate the anti-interference properties of the *C. sakazakii* primers and probes, different bacteria were selected for the SAT system interference test. Twenty-five reference bacterial strains were mixed as group A. Then the above 25 strains were mixed with 6 *C. sakazakii* strains as group B. Total RNA of these strains was extracted as described above and used as template for the SAT reaction system.

Sensitivity of SAT for C. sakazakii

Sensitivity of SAT for C. sakazakii in LB broth

The *C. sakazakii* strains were incubated in LB broth at 37 ℃ for 8 h to about 10^8 CFU/mL. Bacteria were counted on LB agar plate at 37 ℃ overnight. Then, the serial ten-fold dilutions (10^7-10^0 CFU/mL) of bacterial suspensions were obtained using saline solution in triplicate. Total RNA of *C. sakazakii* suspensions (10^7-10^0 CFU/mL) was extracted and used as template for SAT assay.

Sensitivity of SAT for C. sakazakii in powered infant formula

Artificially PIF contaminated with *C. sakazakii* were prepared by directly spiking with *C. sakazakii* under double-blind conditions. The raw infant formula were confirmed to be negative for *C. sakazakii* by selective plating and routine biochemical testing. Then, 100 g of contaminated powdered infant formula were added to 900 mL of buffered peptone water (BPW, pH 7.2) medium (Oxoid, UK) as infant formula broth. The concentration of *C. sakazakii* in each contaminated sample was determined using plating approaches on LB agar plates after overnight incubation at 37 ºC. Then, the serial ten-fold dilutions (10^7-10^0 CFU/mL) were prepared using infant formula liquid medium in triplicate. Total RNA of *C. sakazakii* in the serial dilutions (10^7-10^0 CFU/mL) were extracted and used as template for SAT assay.

Sensitivity of SAT combined with pre-enrichment for C. sakazakii in powdered infant formula

The *C. sakazakii* was incubated in LB broth at 37 ºC for 8 h to about 10^8 CFU/mL. The concentration of bacteria was determined using plating approaches after overnight incubation at 37 ºC. Then, the serial ten-fold serial dilutions (10^3-10^0 CFU/mL, 10^0 CFU /10 mL, 10^0 CFU /100 mL, 10^0 CFU/1000 mL) were prepared using infant formula broth in triplicate and incubated at 37 ºC for 10 h. Every hour, 200 µL of each culture was collected, then total RNA was extracted and SAT assay was performed in triplicate.

Sensitivity Comparison of SAT and Real-Time PCR

To compare the detection limits between SAT and real-time PCR, the *C. sakazakii* incubation, serial dilution and RNA extraction were performed according to the method described in 2.6.1. The *C. sakazakii* was detected using SAT and real-time PCR in the same condition. Furthermore, the serial dilutions (10^7-

10^0 CFU/mL) were autoclaved at 121 °C for 30 min and then stayed at room temperature for 12 h. The *C. sakazakii* was detected to compare the detection of live and killed bacteria SAT and real-time PCR under same condition.

To compare the sensitivity between SAT and real-time PCR with pre-enrichment step, the serial ten-fold dilutions of *C. sakazakii* in BPW were incubated at 37 ºC for 10 h according to the method described in 2.6.3. Then, SAT and real-time PCR were conducted in the same condition.

Results

Specificity and covering of the SAT primers for C. sakazakii

The specificity of the 16/23S rRNA primers was evaluated using 6 *C. sakazakii* strains and 25 reference strains. Only strains of *C. sakazakii* produced positive signal. All the non-*C. sakazakii* strains had no amplification curve before 60 cycles (Fig. 1A). As shown in Fig. 1B, the mixed strains of *C. sakazakii* and non-*C. sakazakii* (group B) produced positive signal while the mixed culture of non-*C. sakazakii* strains (group A) had no amplification signal before 60 cycles. Furthermore, all tested 6 *C. sakazakii* strains could produce amplification signal using the 16/23S rRNA primers (Fig. 1C). These results suggested that the SAT primers designed in this study were specific to *C. sakazakii* identification and had a good covering for the detection of different *C. sakazakii* isolates.

Sensitivity of SAT for the detection of C. sakazakii in pure culture and in powered infant formula

Using pure culture samples artificially contaminated with *C. sakazakii* ten-fold serial dilutions were performed by dissolved into LB broth at 10-10^7 CFU/mL to assay the analytical sensitivity. The results indicated that the limit of detection of samples without pre-enrichment was 10 CFU/mL (Fig. 2A). For PIFs artificially contaminated with *C. sakazakii* ten-fold serial dilutions (10-10^7 CFU/mL), the limit of detection (LOD) of samples without pre-enrichment was 10^2 CFU/mL (Fig. 2B).

Sensitivity Comparison of SAT and Real-Time PCR

Comparison of detection limits between SAT and Real-Time PCR

To compare the detection limits between SAT and Real-time PCR, a series of ten-fold dilutions with 10^0-10^7 CFU/mL of *C. sakazakii* were prepared and analyzed. For *C. sakazakii* in pure culture, the detection limit of SAT and real-time PCR was 10 CFU/mL (Fig. 3A) and 10^3 CFU/mL (Fig. 3B), respectively.

Comparison of detection limits between SAT and Real-Time PCR with enrichment step

As shown in Table 2, PIF containing 10^0 CFU/mL of *C. sakazakii* could be detected successfully by SAT after 3 h enrichment at 37 ºC. For 10^{-1} CFU/mL of *C. sakazakii*, 4 h enrichment was needed. After 8 h enrichment, 10^{-3} CFU/mL of *C. sakazakii* in PIF could be detectable successfully by SAT. Whereas, for

real-time PCR, the detection limit of 10^0 CFU/mL of *C. sakazakii* in PIF was obtained after 5 h enrichment at 37 ºC. For 10^{-1} and 10^{-3} CFU/mL of *C. sakazikii*, 6 h and 10 h enrichment was needed, respectively. The results suggested that the pre-enrichment step was necessary for rapid detection of *C. sakazakii* in food samples (Liu et al. 2006; Mohan Nair and Venkitanarayanan 2006; Ye et al. 2012).

Comparison of viable bacteria detection between SAT and Real-Time PCR

To determine the limit of SAT and real-time PCR in viable bacteria detection, the *C. sakazakii* (10^7-10^0 CFU/mL) was autoclaved at 121 °C for 30 minutes and then stayed at room temperature for 12 h. As shown in Fig. 4, the detection limit of SAT (Fig. 4A) and real-time PCR (Fig. 4B) was 10^6 CFU/mL and 10^3 CFU/mL, respectively. The result indicated that the detection limit of SAT was significantly lower than real-time PCR after autoclaving.

Discussion

It was reported that most of invasive *Cronobacter* infections were in infants who were fed PIFs. So, PIFs were regarded as predominant sources of *Cronobacter* infections (Jason 2012) and the most likely media of transmission associated with hospital outbreaks (van Acker et al. 2001). *Cronobacter* contamination has been repeatedly found in PIF and factories producing it, and in the ingredients used to make PIF and factories processing these ingredients (Parra-Flores et al. 2018; Reich et al. 2010). During the processing, PIF is not sterile. So, some pathogens may be found in PIF, such as *Cronobacter* (Beuchat et al. 2009). If PIF is contaminated with *Cronobacter*, the mixture of PIF and water will become incubators of the bacteria, and then threaten the health of infants. Therefore, rapid, sensitive, simple and accurate techniques for early detection of viable *Cronobacter* spp. in PIFs are highly important and urgent to control the risk of *Cronobacter* related food-borne diseases (Zhu et al. 2012).

To date, a variety of detection and diagnostic approaches for detection of *Cronobacter* spp. have been developed and reported, such as the traditional isolation and biochemical identification directly from culture or after enrichment, or specific screening medium to characterize samples potentially contaminated (Cai et al. 2013). However, the current approved method for detection of *Cronabacter* spp. in PIFs is time-consuming, laborious and expensive due to the requirement of enrichment culture, incubation on selective media and phenotypic characterization (Druggan and Iversen 2009; International Organization for Standardization 2006b; Turcovsky et al. 2011).

At present, there are several rapid detection methods especially the molecular methods based on PCR amplification. DNA targeting molecular methods such as PCR, real-time PCR and LAMP, had many advantages over conventional approaches. Jaradat et al. (2009) indicated that *Cronobacter* spp. have high diversity and share a lot of phenotypic traits with other *Enterobacteriaceae* members. So, different chromogenic, biochemical, and molecular techniques was used to confirm the identity of this pathogen. The results revealed that 16s rRNA sequencing should be used as a final confirmation and is pivotal to eliminate the doubts shed by the inability of other methods for identification and confirmation of the

identity of the *Cronobacter spp.* Cai et al. (2013) developed an assay integrating real-time PCR and high resolution melting (HRM) analysis to specifically detect and rapidly identify *Cronabacter* spp. in PIF using *OmpA* gene. The method was proved to be sensitive with a detection limit of 10^2 CFU/mL without pre-enrichment, and highly concordant with the accuracy of 100% compared with ISO-IDF 22964 in 89 real samples (Cai et al. 2013). This method can need detect *Cronobacter* spp. in about 24 h, which is far quicker than the up to 7 days of the currently routine procedure.

In the present study, we have developed a SAT method targeting 16s/23s rRNA for specific detection and rapid identification of viable *Cronobacter* spp. in PIFs and other foodstuffs. The SAT method developed in this study can detect *Cronobacter* spp. in about 1 h 20 min without pre-enrichment, which is far quicker than the method developed in previous studies (Cai et al. 2013). Compared with other detection methods, the SAT method developed in this study has some advantages, such as (1) the amplification reaction is performed at a constant temperature, which can reduce the requirement for needing equipment; (2) the detection target is RNA, which has much higher concentration than DNA; (3) the amplification rate of RNA is much higher than that of DNA, which can shorten the detection time; (4) RNA is quickly degraded in dead bacteria, which can accurately distinguish viable *Cronobacter* spp. from the dead one; (5) the upstream and downstream primers and the probe is highly specific, which greatly improves the specificity of detection.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflict of interest. All authors have read and approved the final manuscript.

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Authors' contributions

RW, XL and FX conceived and designed the experiments; XS, XG, GW, XW, WM, LZ and QL performed the experiments; RW, XS and FX analyzed the data; RW, XL and FX wrote the paper.

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Tables

Table 1. Bacterial species and strains used in RT-LAMP-SAT assay

Species/Strains	Strains number	Source
Cronobacter malonaticus	CICC21551	China Center of Industrial Culture Collection
Cronobacter dublinensis	CICC21564	China Center of Industrial Culture Collection
Cronobacter universalis	CICC21570	China Center of Industrial Culture Collection
Cronobacter muytjensii	CICC23943	China Center of Industrial Culture Collection
Cronobacter turicensis	CICC24178	China Center of Industrial Culture Collection
Cronobacter condimenti	CICC24184	China Center of Industrial Culture Collection
Citrobacter Freundii	ATCC8090	American Type Culture Collection
Enterobacter cloacae	CICC10011	China Center of Industrial Culture Collection
Enterococcus faecalis	CICC10396	China Center of Industrial Culture Collection
Escherichia coli	ATCC25922	American Type Culture Collection
Klebsiella peneumoniae	CICC21519	China Center of Industrial Culture Collection
Listeria monocytogenes	CMCC54001	National Center for Medical Culture Collections
Listeria welshimeri	CICC21672	China Center of Industrial Culture Collection
Listeria innocua	CICC 10417	China Center of Industrial Culture Collection
Proteus vulgaris	CMCC(B)49027	National Center for Medical Culture Collections
Pseudomonas aeruginosa	ABCC0927	China Center of Industrial Culture Collection
Salmonella paratyphi B	CICC21495	China Center of Industrial Culture Collection
Salmonella paratyphi-A	CICC21501	China Center of Industrial Culture Collection,
Salmonella enteritidis	CMCC(B)5004	National Center for Medical Culture Collections
Salmonella typhimurium	CMCC(B)50115	National Center for Medical Culture Collections
Shigella flexneri	CICC10865	China Center of Industrial Culture Collection
Shigella dysenteriae	CICC10865	China Center of Industrial Culture Collection
Shigella sonnei	CICC 21535	China Center of Industrial Culture Collection
Shigella castellani	CMCC(B)51572	National Center for Medical Culture

		Collections
Staphylococcus aureus	ATCC25923	American Type Culture Collection
Staphylococcus xylosus	CICC20237	China Center of Industrial Culture Collection
Staphylococcus epidermidis	CICC10294	China Center of Industrial Culture Collection
Vibrio parahaemolyticus	CICC21528	China Center of Industrial Culture Collection
Vibrio parahaemolyticus	ATCC17802	American Type Culture Collection
Vibrio vulnificus	CICC21615	China Center of Industrial Culture Collection
Yersinia pseudotuberculosis	CICC21669	China Center of Industrial Culture Collection

Table 2 Comparison of pre-enrichment time between SAT and Real-Time PCR

concentration（CFU/mL）		1h	2h	3h	4h	5h	6h	7h	8h	9h	10h
SAT	Blank	-	-	-	-	-	-	-	-	-	-
	10^{-3}	-	-	-	-	-	-	-	+	+	+
	10^{-2}	-	-	-	-	-	-	-	+	+	+
	10^{-1}	-	-	-	+	+	+	+	+	+	+
	10^{0}	-	-	+	+	+	+	+	+	+	+
	10^{1}	-	+	+	+	+	+	+	+	+	+
	10^{2}	+	+	+	+	+	+	+	+	+	+
	10^{3}	+	+	+	+	+	+	+	+	+	+
Real-timePCR	Blank	-	-	-	-	-	-	-	-	-	-
	10^{-3}	-	-	-	-	-	-	-	-	-	-
	10^{-2}	-	-	-	-	-	-	-	-	-	-
	10^{-1}	-	-	-	-	-	-	-	+	+	+
	10^{0}	-	-	-	-	+	+	+	+	+	+
	10^{1}	-	-	-	+	+	+	+	+	+	+
	10^{2}	-	-	+	+	+	+	+	+	+	+
	10^{3}	+	+	+	+	+	+	+	+	+	+

Figures

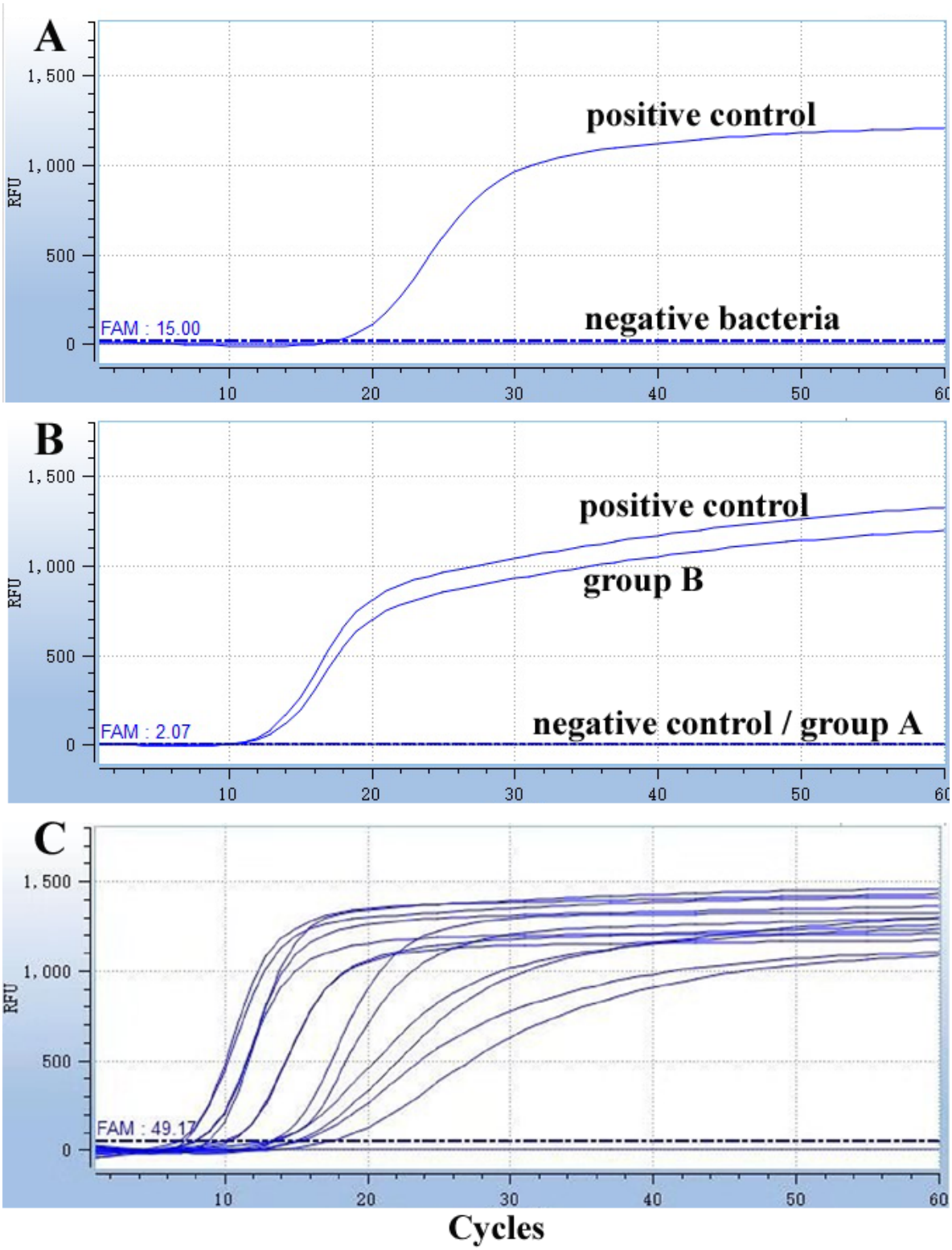

Figure 1

SAT analysis for all bacterial strains. (A) SAT amplification for positive control (C. sakazikii) and negative bacteria (non-C. sakazikii). (B) SAT amplification for positive control (C. sakazikii) , group B (the mixed strains of C. sakazikii and non-C. sakazikii strains) and group A (the mixed culture of non-C. sakazakii strains). (C) SAT amplification for 6 C. sakazakii strains.

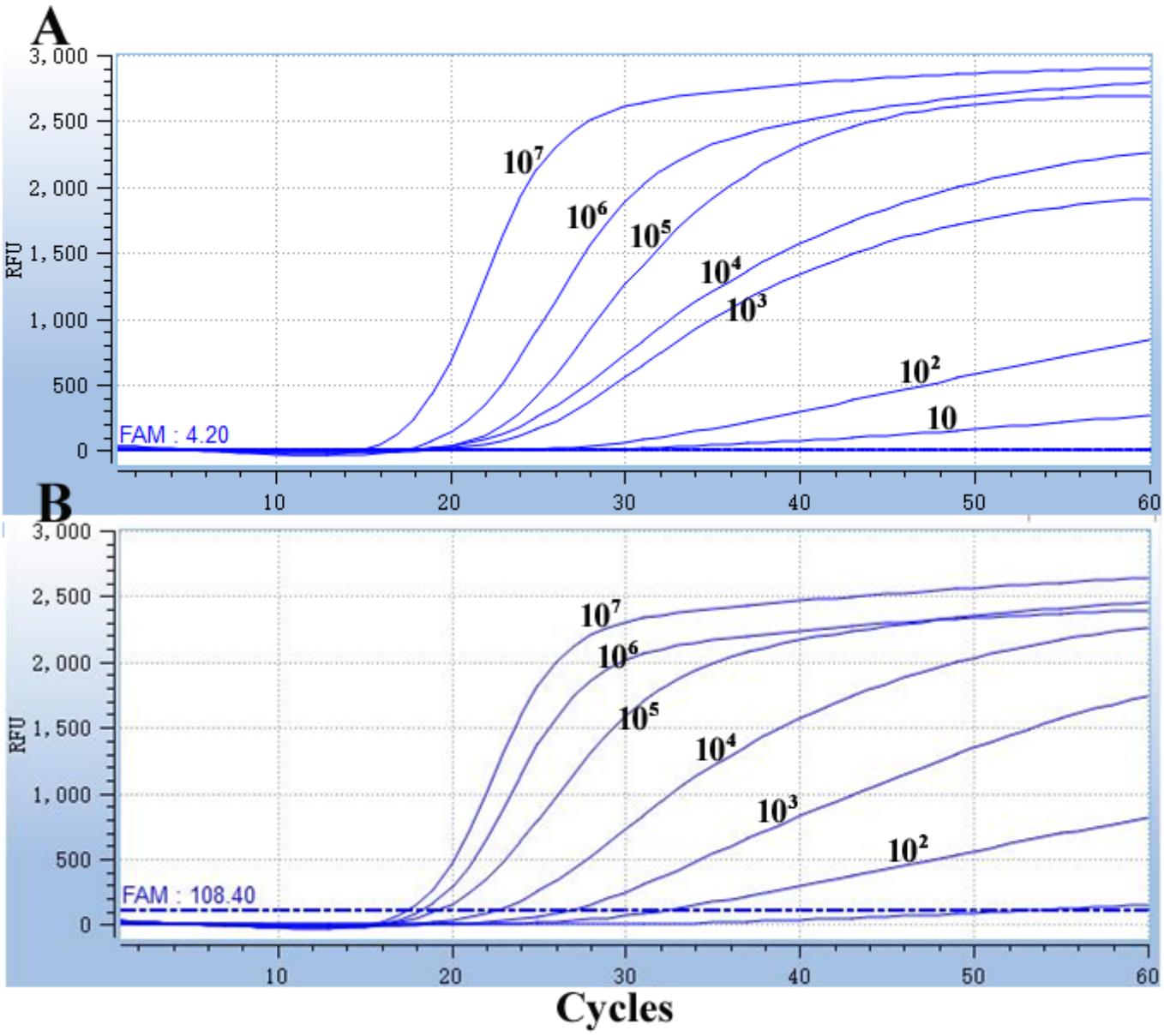

Figure 2

SAT analysis of 10-fold serial dilution of C. sakazakii in pure culture and in powdered infant formula. (A) Pure culture. (B) Powdered infant formula.

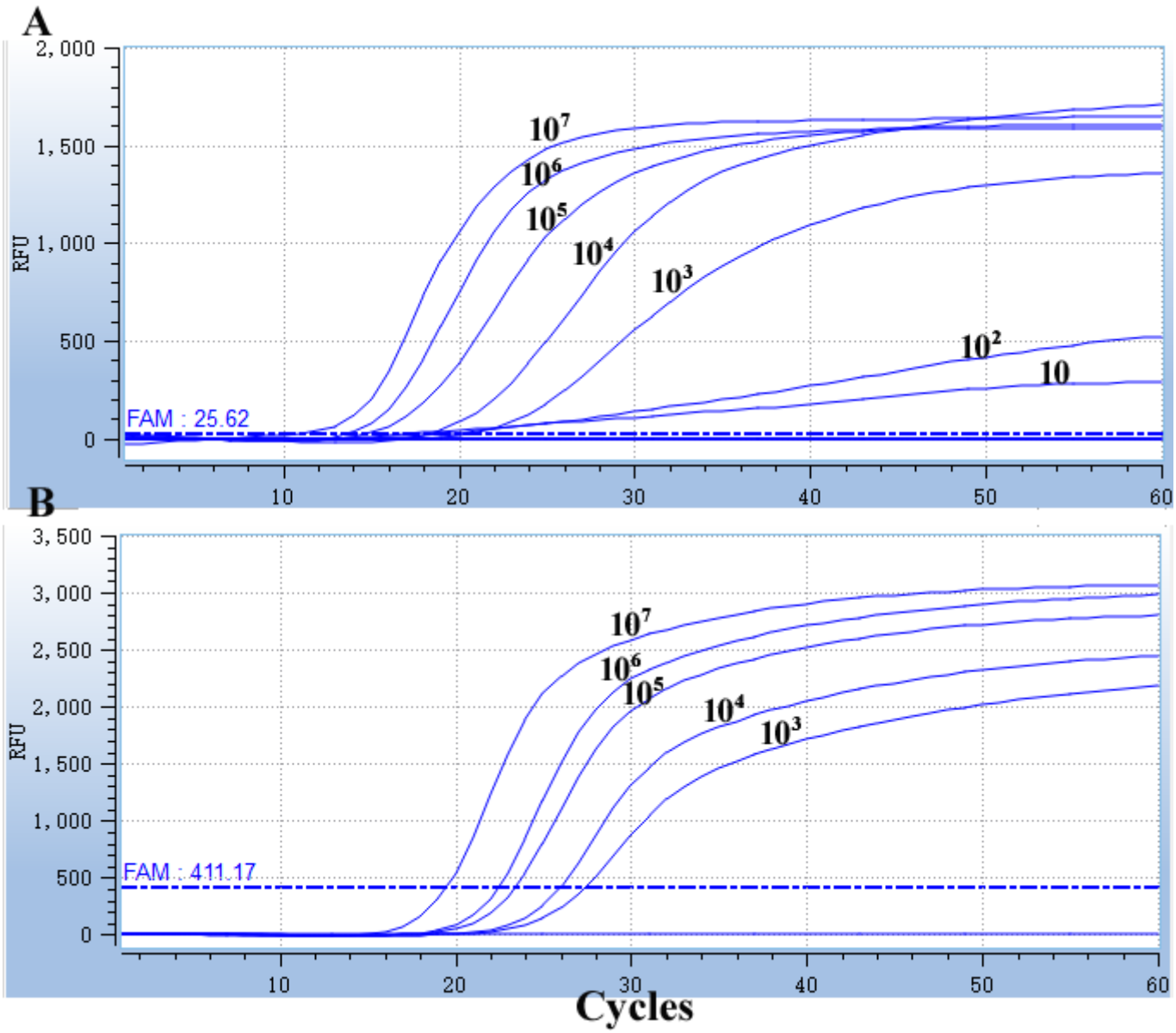

Figure 3

The detection limits of SAT and Real-time analysis without enrichment. (A) SAT analysis. (B) Real-time PCR analysis.

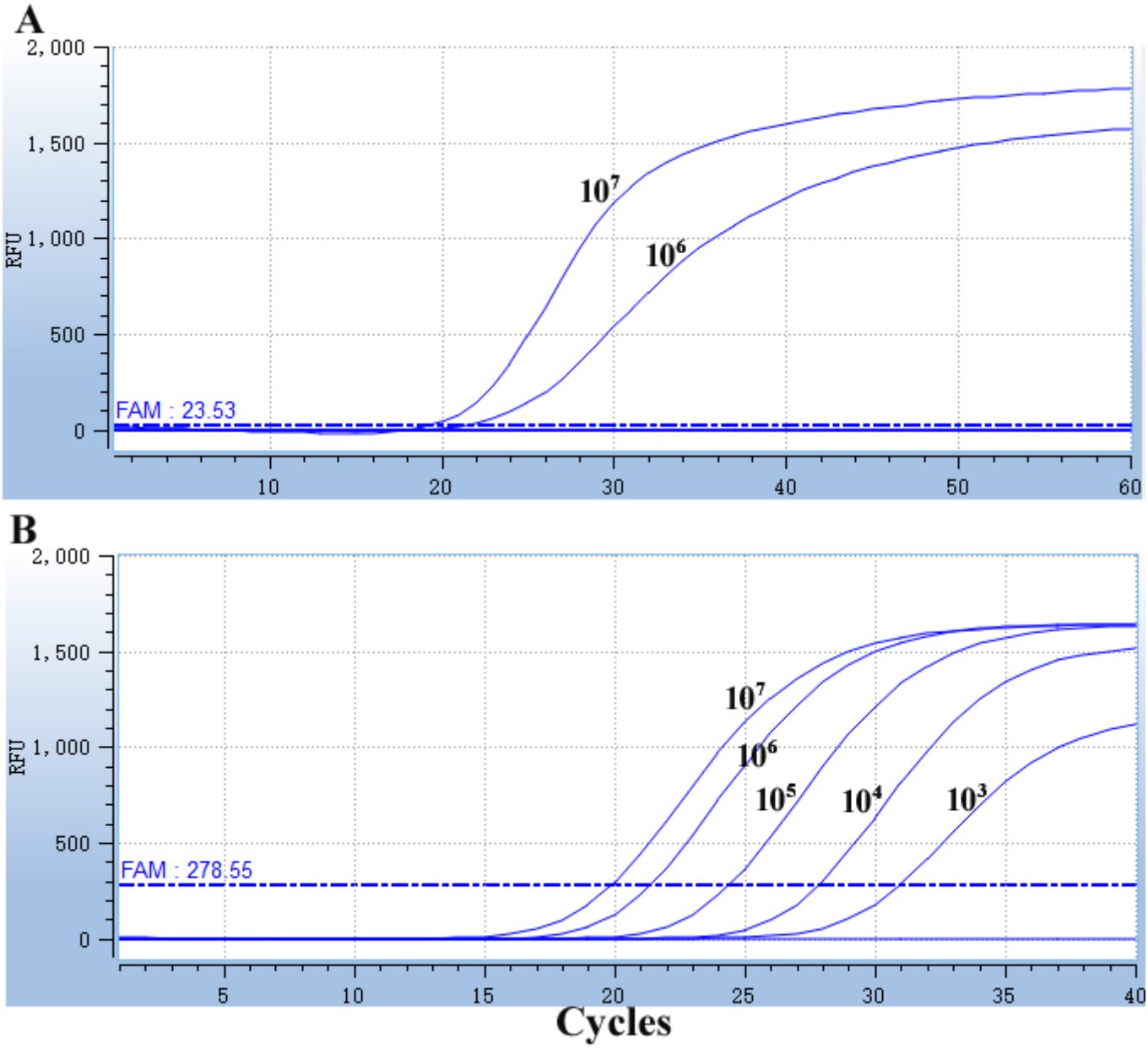

Figure 4

The viable bacteria detection of SAT and Real-time PCR analysis. (A) SAT analysis. (B) Real-time PCR analysis.