Electricity-free amplification and visual detection of *Cronobacter* species in powdered infant formula

Shiqian Fu,* Yujun Jiang,* Xue Qin, Tao Yang, Sihan Chen, Xinyan Yang, Wei Zhang, Yanyan Qu, and Chaoxin Mant

Key Laboratory of Dairy Science, Ministry of Education, College of Food Science, Northeast Agricultural University, Harbin 150030, China

ABSTRACT

Due to the lack of electricity and thermostatic instruments in certain settings for convenient detection of *Cronobacter* species in powdered infant formula (PIF), a novel investigation was conducted to establish an electricity-free visual detection system for rapid detection of *Cronobacter* species in PIF. This system included a portable electricity-free heater that could use the exothermic reaction of calcium oxide and water and 3 kinds of phase change materials to supply 3 constant temperatures for immunomagnetic separation, DNA extraction, and loop-mediated isothermal amplification assay. Meanwhile, the amplified reaction combined with hydroxynaphthol blue could achieve rapid visual detection. Primers designed based on the 16S–23S ribosomal RNA internal transcribed spacer were used in loop-mediated isothermal amplification to specifically monitor *Cronobacter* species, and the detection limit can reach 4.2×10^2 cfu/g in PIF by an electricity-free heater in 2 h 30 min. Moreover, 2 h of pre-enrichment was necessary when the level of the PIF samples with *Cronobacter* spp. was 10^0 cfu/g. The stability of the system was evaluated in ambient temperature at 4°C, 25°C, and 37°C. The results suggested that the electricity-free heater can maintain 3 constant temperatures to support different processes. Therefore, this amplification and visual system is applicable for use in many fields for rapid and specific detection of *Cronobacter* species in PIF.

Key words: electricity-free visual detection, immunomagnetic separation, loop-mediated isothermal amplification, powdered infant formula, *Cronobacter* species

INTRODUCTION

Cronobacter spp., previously called *Enterobacter sakazakii*, mainly infect newborns and infants and can cause meningitis, necrotizing enterocolitis, and bacteremia with a fatality rate of about 40 to 80% (Healy et al., 2010; Yan and Fanning, 2015). Powdered infant formula (PIF) has been recognized as the main source of infection (Song et al., 2016), and the delayed detection and report of contaminated pathogens in PIF could cause outbreaks of foodborne disease (Norberg et al., 2012). Consequently, a simple and rapid detection method is extremely important in resource-limited settings. The immunochromatographic strip has been widely applied to detect pathogens because of its simplicity, rapidness, and no need for equipment (Huang et al., 2016; Luo et al., 2017), whereas it has some limitations in sensitivity and specificity. Nucleic acid detection as a rapid detection method is very efficient for detecting pathogens. However, most nucleic acid detecting techniques have been restricted to laboratories rather than the limited conditions where need is foremost (Pecchia and Lio, 2018).

Loop-mediated isothermal amplification (LAMP) is maintained at 65°C for 1 h and has high specificity and sensitivity without trained persons, costly equipment, or thermal cycles (Yan et al., 2014; Yin et al., 2016); the operation process is simple and much less sensitive to inhibitors than PCR assay (Zhao et al., 2017). Furthermore, LAMP products can be observed by direct turbidity, fluorescent dye, and immunochromatographic strips (Singleton et al., 2014; Wang et al., 2017).

The LAMP assay requires a heat source to complete amplified reaction. To eliminate the demand for electric instruments in limited conditions, some electricity-free heaters have been designed to maintain temperature of LAMP reaction in recent years (Labarre et al., 2010; Sema et al., 2015). These electricity-free heaters use the heat release of chemical reactions combined with phase change material (PCM) to regulate temperature (Labarre et al., 2011; Liao et al., 2016). For example,

Received September 26, 2019.

Accepted March 9, 2020.

*These authors contributed equally to this work.

†Corresponding author: mxcwh2006@qq.com

Curtis et al. (2012) designed a noninstrumented nucleic acid heater for point-of-care detection of HIV-1, and the LAMP assay for detection of *E. coli* has been conducted using a self-heating cartridge (Liu et al., 2011). Additionally, the analysis of on-site detection revealed that LAMP products integrating with these electricity-free devices could be simultaneously detected by immunochromatographic strip and fluorescent dyes to obtain visual detection results (Hatano et al., 2010; Singleton et al., 2014). However, all aforementioned devices lack the capabilities of bacteria pre-enrichment and DNA extraction, despite various DNA extraction methods that have been developed (Abdelhai et al., 2016). These methods are generally conducted in the laboratory; for example, centrifuge procedure for concentration of the bacteria requires electricity, and could not be completed in limited conditions. Magnetic nanobead-antibody complex (**MNB-Ab**) has been applied to perform immunomagnetic separation (**IMS**) in samples (Wang et al., 2011; Sun et al., 2015), which could selectively concentrate target bacteria at a constant temperature. Hence, IMS combined with LAMP assay is more suitable for electricity-free detection of pathogenic bacteria.

In our investigation, an electricity-free heater as reaction equipment that uses 3 kinds of PCM to regulate different constant temperatures was introduced for rapid detection of *Cronobacter* spp. in PIF. The process was divided into 3 steps including the IMS, DNA extraction, and LAMP-hydroxynaphthol blue (**HNB**) assay. The HNB was added into the reaction mixture in advance to avoid contamination and achieve visual detection. Thus, an electricity-free amplification and visual detection system was established for *Cronobacter* spp. in PIF in resource-limited settings.

MATERIALS AND METHODS

Portable Electricity-Free Heater

The portable electricity-free heater we designed obtained heat resource from the exothermic reaction of calcium oxide and water, and the schematic diagram is shown in Figure 1. Three kinds of PCM (Rubitherm, Berlin, Germany) with different heating capacity and melting temperature were used to maintain required temperature for the whole detection process, and the PCM maintained temperatures of 35°C, 65°C and 100°C, respectively, which contributed to the steps of IMS, DNA extraction, and LAMP reaction. A vacuum cup was employed for thermal insulation. The heater consisted of 2 chambers, one containing the exothermic reaction and the other containing an aluminum box with PCM, which had a hole in the center. The size of hole was suitable for a 200- μ L PCR tube and 2-mL epoxy epoxide tube, respectively, and the different steps in the same electricity-free heater were conducted in the process of detection by selecting the aluminum box with the desired size of hole and adding different weights of calcium oxide and water, combining with PCM for the required melting temperature. Then the tube was embedded in PCM through the hole. One type K (Fluke Process Instruments, Shanghai, China) thermocouple connected with a digital thermometer was inserted in PCM which can measure reaction temperature in real time.

Preparation of MNB-Ab

The mouse anti-*Cronobacter* spp. monoclonal antibody (Shanghai Huiyun, Shanghai, China) was conjugated with carboxyl magnetic nanobeads (**MNB**)

Figure 1. Schematic diagram of the portable electricity-free heater. (A) The appearance of the electricity-free heater. (B) The transection of the internal structure of the electricity-free heater.

forming covalent conjugates via amide bonds using carbodiimide hydrochloride according to the manufacturer's instruction (Sigma, St. Louis, MO). The preparation procedures of MNB-Ab are described as follows: 50 μ L of carboxyl MNB (10 mg/mL) was washed with 560 μ L of 2-(*N*-morpholino)ethanesulfonic acid (MES) solution (pH 6.5, 15 mM) and magnetic separation for 2 min, then supernatant was removed. The process was repeated 3 times. Then, carboxyl MNB were suspended in 400 μ L of MES solution, and underwent ultrasonic treatment for 10 min at 200 W, and magnetic separation for 2 min. The supernatant was removed and the sediment was suspended again in 400 μ L of MES solution. Then 60 μ L of carbodiimide hydrochloride solution (10 mg/mL) was mixed and incubated at room temperature under shaking for 30 min; next, the mixtures were washed 3 times using MES solution and MEST solution (15 mM MES, 0.02% Tween 20, pH 6.0), respectively, followed by magnetic separation and removing the supernatant. Subsequently, the carboxyl MNB were blended with 400 μ L of MEST solution and 80 μ L of antibody (2.6 mg/mL) to incubate for 12 h at room temperature under shaking so that the antibody could link with carboxyl MNB; then, the mixtures were washed 5 times using 400 μ L of PBST solution (10 mM PBS, 0.02% Tween 20, pH 6.0) and 400 μ L of PBS solution (10 mM, pH 7.3), respectively, to wash away the antibody that did not conjugate with carboxyl MNB. After magnetic separation and elimination of supernatant, the sediment was finally resuspended in 500 μ L of PBS solution as MNB-Ab for IMS.

Bacterial Strain Culturing and Sample Preparation

A total of 44 bacterial strains including 19 *Cronobacter* strains and 25 non-*Cronobacter* strains are listed in Table 1. All *Cronobacter* strains were cultured in 20 mL of trypticase soy broth at 37°C for 14 h. The other 25 bacterial strains were incubated in nutrient broth at 37°C under shaking (200 rpm) for 14 h to extract DNA. *Cronobacter sakazakii* ATCC 29544 was used as a sample to detect the sensitivity in PIF and the stability of the device. To determine the concentration of *C. sakazakii*, 100 μ L of serial 10-fold dilution of enriched *C. sakazakii* was added to trypticase soy agar plates and incubated at 37°C for 18 h before enumeration. The PIF samples purchased from supermarkets were tested according to the method of the US Food and Drug Administration to determine that the samples were not contaminated with *Cronobacter* spp. (Kim et al., 2016). Then 10 g of PIF was homogenized in 90 mL of sterile PBS solution, and 9 mL of the PIF mixture was inoculated with 1 mL of above-mentioned 10-fold dilution of *C. sakazakii* culture, respectively. The PIF

contaminated by *C. sakazakii* ATCC 29544 underwent the processes of IMS, DNA extraction, and LAMP assay; meanwhile, the uncontaminated PIF served as a negative control.

Process of IMS, DNA Extraction, and LAMP Assay for *Cronobacter* spp. in PIF with a Portable Electricity-Free Heater

Process of IMS. The weight of PCM (35°C) that was added to the upper chamber was fixed at 8 g, and the proportion of calcium oxide and water was optimized within the trial and repeated 3 times. Then, the mixture of 2 reagents was introduced into the bottom chamber by stirring. Meanwhile, 400 μ L of PIF contaminated by different concentrations of bacteria was blended with 50 μ L of MNB-Ab in a 2-mL epoxy epoxide tube. Then the epoxy epoxide tube was put into PCM through the hole to capture strains by IMS, and was incubated for 1 h at 35°C with a portable electricity-free heater. Next, after magnetic separation for 2 min, the supernatant was removed and mixtures were washed 3 times by PBS solution. After magnetic separation, the supernatant was removed; then, the captured bacteria strains were resuspended in 100 μ L of PBS solution and placed on a magnet for 1 min. The total process was repeated 4 times. The first time was conducted to remove supernatant to prepare DNA extraction, and the other 3 times were to conducted to calculate the concentration of bacteria in PIF by the plate counting method.

Process of DNA Extraction. The weight of PCM (100°C), which was added to the upper chamber, fixed at 9 g. The optimal ratio of calcium oxide and water was determined by repeating 3 times; then, the mixture of 2 materials was added to the bottom chamber by stirring. The above-mentioned captured bacteria in a 2-mL epoxy epoxide tube with different concentrations were mixed with 30 μ L of DNA lysis buffer [pH 8.0, 10 mM Tris, 4.5% (vol/vol) Triton X-100, 4.5% (vol/vol) Tween 20], respectively, then the epoxy epoxide tube was embedded into PCM through the hole and boiled for 3 min at 100°C to complete DNA extraction with the portable electricity-free heater. Finally, the mixtures as DNA template were injected into the LAMP reaction.

Process of LAMP Assay. The weight of PCM (65°C) that was added to the upper chamber fixed at 10 g as an invariant, and the proportion of calcium oxide and water was tested to obtain the optimal ratio. Two reagents were added to the bottom chamber by stirring, repeating the optimization experiment 3 times. The LAMP mixtures in the 200- μ L PCR tube were inserted into PCM; then, the LAMP reaction was

Table 1. Strains of *Cronobacter* species (*Cronobacter* spp.) and non-*Cronobacter* spp. used for loop-mediated isothermal amplification in an electricity-free heater¹

Bacterial species	Strain	Source	HNB	AGE
<i>Cronobacter</i> strain				
<i>Cronobacter sakazakii</i>	ATCC 29544	National Institutes for Food and Drug Control	+	+
12 <i>C. sakazakii</i> ²	Own collection	Isolated from powdered infant formula and baby foods	+	+
<i>C. malonaticus</i>	DSM 18702	Deutsche Sammlung von Mikroorganismen und Zellkulturen	+	+
<i>C. turicensis</i>	DSM 18703	Deutsche Sammlung von Mikroorganismen und Zellkulturen	+	+
<i>C. mytilensis</i>	ATCC 51329	American Tissue Culture Collection	+	+
<i>C. universalis</i>	NCTC 9529	National Collection of Type Cultures	+	+
<i>C. dublinensis</i>	CICC 18705	China Center of Industrial Culture Collection	+	+
<i>C. condimenti</i>	LMG 26250	Belgian Coordinated Collections of Microorganisms	+	+
Non- <i>Cronobacter</i> strain				
<i>Enterobacter cloacae</i>	ATCC 3503	Fujian Center For Disease Control and Prevention	–	–
<i>Enterobacter aerogenes</i>	ATCC 13048	National Institutes for Food and Drug Control	–	–
<i>Listeria monocytogenes</i>	CMCC 54004	National Institutes for Food and Drug Control	–	–
<i>L. monocytogenes</i>	CMCC 54006	National Institutes for Food and Drug Control	–	–
<i>L. monocytogenes</i>	CMCC 54002	National Institutes for Food and Drug Control	–	–
<i>Listeria innocua</i>	ATCC 33090	National Institutes for Food and Drug Control	–	–
<i>Listeria welshimeri</i>	ATCC 43548	National Institutes for Food and Drug Control	–	–
<i>Salmonella typhimurium</i>	ATCC 14028	National Institutes for Food and Drug Control	–	–
<i>Salmonella enteritidis</i>	ATCC 13076	National Institutes for Food and Drug Control	–	–
<i>Salmonella choleraesuis</i>	CMCC 50018	National Institutes for Food and Drug Control	–	–
<i>Salmonella paratyphi A</i>	CMCC 50093	National Institutes for Food and Drug Control	–	–
<i>Salmonella enterica paratyphi B</i>	CMCC 50094	National Institutes for Food and Drug Control	–	–
<i>Salmonella arizonae</i>	CMCC 47020	National Institutes for Food and Drug Control	–	–
<i>Salmonella dublin</i>	CMCC 50092	National Institutes for Food and Drug Control	–	–
<i>Salmonella thompson</i>	CMCC 50120	National Institutes for Food and Drug Control	–	–
<i>Shigella flexneri</i>	CMCC 51572	Fujian Center For Disease Control and Prevention	–	–
<i>Staphylococcus aureus</i>	CMCC 26075	National Institutes for Food and Drug Control	–	–
<i>Staph. aureus</i>	ATCC 13565	National Institutes for Food and Drug Control	–	–
<i>Staphylococcus epidermidis</i>	ATCC 26069	China General Microbiological Culture Collection Center	–	–
<i>Escherichia coli</i> O157:H7	ATCC 25922	National Institutes for Food and Drug Control	–	–
<i>Bacillus cereus</i>	CMCC 63303	Fujian Center For Disease Control and Prevention	–	–
<i>Vibrio parahaemolyticus</i>	ATCC 17802	National Institutes for Food and Drug Control	–	–
<i>Franconibacter helveticus</i>	LMG 23732	Deutsche Sammlung von Mikroorganismen und Zellkulturen	–	–
<i>Franconibacter puberis</i>	LMG 24057	Deutsche Sammlung von Mikroorganismen und Zellkulturen	–	–
<i>Siccibacter turicensis</i>	LMG 23730	Deutsche Sammlung von Mikroorganismen und Zellkulturen	–	–

¹HNB = hydroxynaphthol blue; AGE = agarose gel electrophoresis. + = positive result; – = negative result.²12 *C. sakazakii*. These strains were isolated from powdered infant formula and baby foods, and encoded as CE13, CE15, CE16, CE28, CE29, CE32, CE52, CE55, CE56, CE62, CE63, and CE64.

performed with a portable electricity-free heater. In this investigation, LAMP primer sequences targeting the internal transcribed spacer (ITS) of *Cronobacter* spp. were described previously, which including 2 outer primers (**F3** and **B3**), 2 inner primers (**FIP** and **BIP**), and 2 loop primers (**LF** and **LB**; Liu et al., 2009). The optimal conditions of the LAMP assay were determined by the portable electricity-free heater, including reaction time and temperature, proportion of primers, and the concentration of reagents. The reagents consisted of dNTP, Bst 2.0 WarmStart DNA polymerase (New England BioLabs, Ipswich, MA), and MgSO₄.

Visual Detection of Amplification Products. The LAMP products were visually detected by color changing of HNB, and a positive result with color of sky blue was observed following the addition of HNB in LAMP reaction beforehand, whereas a negative result showed violet. To prove the reliability of this method, 2% agarose gel electrophoresis (AGE) and optical density (OD) detection was conducted. The results were valued according to the electrophoretic band using AGE and the ratio of OD value tested in different wavelengths of light by a microplate reader.

Specificity and Sensitivity Test of LAMP for *Cronobacter* spp.

Specificity of LAMP Assay for *Cronobacter* spp. The DNA from 19 *Cronobacter* spp. strains and 25 non-*Cronobacter* spp. strains were used to determine the specificity of the LAMP assay. The LAMP reaction was conducted in a portable electricity-free heater. The results were observed by color changing of HNB and electrophoretic band under UV light after AGE.

Sensitivity for *Cronobacter* spp. in Powdered Infant Formula. The IMS, DNA extraction, and LAMP assay were performed by using a portable electricity-free heater and conventional electric device, respectively. The above-mentioned DNA extracted from different concentrations of *C. sakazakii* ATCC 29544 in PIF served as positive templates, and sterile distilled water was used as a negative control to carry out the LAMP assay, and the amplified products were detected by HNB and 2% AGE to determine the sensitivity. At the same time, the traditional PCR method was carried out, and LAMP outer primers (F3 and B3) were used as PCR primers to compare the detection sensitivity.

Interference and Stability Test for LAMP with Portable Electricity-Free Heater

To test the interference of background microflora in the PIF samples, *Listeria monocytogenes* CMCC 54004, *Salmonella typhimurium* ATCC 14028, *Escherichia coli*

O157:H7 ATCC 25922, and *Bacillus cereus* CMCC 63303 were cultured to achieve different concentrations of strains by serial 10-fold dilution. Next, the above non-*Cronobacter* spp. were mixed with *C. sakazakii* in equal volumes to obtain contaminated PIF. The final concentrations of non-*Cronobacter* spp. ranged from 10⁰ to 10⁸ cfu/g, whereas that of *C. sakazakii* fixed at 10⁷ cfu/g. Then DNA was extracted from the mixtures and was used as a template for LAMP-HNB and LAMP-AGE detection with a portable electricity-free heater. Additionally, the stability of the heater and detection limit was tested with electricity-free amplification and visual detection system at 4°C, 25°C, and 37°C, respectively.

RESULTS AND DISCUSSION

Optimization of IMS, DNA Extraction, and LAMP Assay

Process of IMS. The components of PIF were complicated, which was unfavorable for DNA extraction, so pre-concentration and purification for targeted bacteria were necessary. Even though IMS have been applied to concentrate the pathogenic bacteria, a previous study only conducted IMS by utilizing conventional electric heaters, such as a shaking table and incubator (Yang et al., 2013). In this assay, an electricity-free heater was introduced to perform IMS, and the weight of PCM (35°C) and calcium oxide fixed at 8 and 15 g, and the volume of water was added at 1.4, 1.6, 1.8, and 2.0 mL, respectively. The temperature profile of the electricity-free heater was generated by calculating the mean and standard deviation of temperature (Figure 2). The results indicated that 15 g of calcium oxide and 1.6 mL of water combining with 8 g of PCM (35°C) could maintain 60 min at 35°C to capture the *Cronobacter* spp. by IMS. At this temperature, the MNB-Ab was used to capture the bacteria to form conjugate that can be separated by a magnet. A 2-mL epoxy epoxide tube was taken out from heater at 67 min including warm-up time. Therefore, the method was more appropriate for resource-limited settings compared with the previous method, and electricity and complicated instruments were not required.

Process of DNA Extraction. To detect the captured bacteria, DNA was needed as template to carry out nucleic acid amplification, and various DNA extraction methods were reported (Wu et al., 2014; Bletz et al., 2015). However, the DNA kit was expensive, time consuming, and complicated. The boiling method was low cost and rapid, but centrifuge was needed. The phenol-chloroform isoamyl alcohol method and glass bead method needed steps of centrifugation and vor-

tex. Hence, these techniques were not appropriate for on-site detection. In this assay, DNA lysis buffer was added to the electricity-free heater to solve the above problems. The weights of PCM (100°C) and calcium oxide were fixed at 9 and 42 g, whereas the volume of water was added at 5, 6, 7, 7.5 mL, respectively. The temperature profile of electricity-free heater was plotted by calculating the mean and standard deviation of temperature (Figure 3). The results showed that 42 g of calcium oxide and 7 mL of water together with 9 g of PCM (100°C) could maintain 30 min at 100°C, which aimed to extract DNA of *Cronobacter* spp. by adding the lysis buffer method. A 2-mL epoxy epoxide tube was removed from the heater after 15 min including warm-up time; thus, the temperature profile suggested that a portable electricity-free heater could complete the DNA extraction. The method was simple, rapid,

and low cost, and did not need an electric device; meanwhile, the process of IMS and DNA extraction could be completed with a portable electricity-free heater in 1 h 30 min.

Process of LAMP Assay. The weights of PCM (65°C) and calcium oxide were fixed at 10 and 30 g, respectively, and the volume of water was added at 4, 5, 6, and 7 mL. The temperature profile was generated similarly as above. Figure 4 illustrated that 30 g of calcium oxide and 5 mL of water combined with 10 g of PCM (65°C) could keep 45 min at 65°C for the LAMP assay, so the 200- μ L PCR tube was taken out from the heater approximately at 60 min including preheating time. After optimization of LAMP reaction with the portable electricity-free heater, the optimal LAMP assay was performed in 25 μ L of a mixture containing 2 μ L of extracted DNA template after IMS, 1.6

Figure 2. The optimization of the proportion of calcium oxide and water combining with phase change material (PCM, 35°C). The weights of PCM and calcium oxide were 8 and 15 g, respectively. A–D: the volume of water was 1.4, 1.6, 1.8, and 2.0 mL, respectively. Data shown as mean $\pm$ SD, $n = 3$.

Figure 3. The optimization of the proportion of calcium oxide and water combining with phase change material (PCM, 100°C). The weights of PCM and calcium oxide were 9 and 42 g, respectively. A–D: the volume of water was 5, 6, 7, and 7.5 mL, respectively. Data shown as mean $\pm$ SD, $n = 3$.

μM each of the FIP and BIP primers, 0.2 μM each of the F3 and B3 primers, 0.8 μM each of the LF and LB primers, and 0.8 μL of Bst 2.0 WarmStart DNA polymerase (8 U/ μL), 2.5 μL of 10 $\times$ ThermoPol Buffer (200 mM Tris-HCl, 500 mM KCl, 20 mM MgSO_4 , 100 mM $(\text{NH}_4)_2\text{SO}_4$, 1% Tween 20 (pH 8.8) at 25°C), 2 mM dNTP, 4 mM MgSO_4 , 4.2 μL of double-distilled H_2O , and 150 μM HNB, which could attain the visual detection of *Cronobacter* spp. in PIF. Subsequently, the system was performed at 65°C for 45 min. The LAMP assay combined with an electricity-free heater did not require skilled personnel and costly equipment; thus, it could be completed under limited conditions.

Among nucleic acid amplification detection, the LAMP reaction only required a constant temperature, and various visualized detection methods for LAMP products have been developed, such as lateral flow dipsticks, Quant-iT PicoGreen, and SYBR Green I (Wang et al., 2015; Syedmoradi et al., 2017). However, the opening reaction tube exposed in air made the products be easily contaminated and gave rise to false-positive results. The calcein and MnCl_2 assay showed a series

of partial inhibition and color changing was difficult to observe. Our HNB detection method enabled instant visualization of LAMP products without complicated equipment needing electricity. Additionally, HNB was introduced into the LAMP reaction beforehand; thus, it could avoid cross-contamination of LAMP reagents and amplification products.

Establishment of the Electricity-Free Amplification and Visual Detection System

Although the methods of IMS, DNA isolation, and LAMP assay have been developed to detect pathogens (Sun et al., 2015), they needed to be accomplished in electricity-settings or laboratories. The previous study also revealed that the LAMP assay was hardly conducted by electricity-free heaters in limited conditions (Kubota et al., 2011). In this investigation, the IMS, DNA extraction, and LAMP assay with a portable electricity-free heater could maintain the required temperature and time as described above. Although the reaction process could be conducted, the visual detection

Figure 4. The optimization of the proportion of calcium oxide and water combining with phase change material (PCM, 65°C). The weights of PCM and calcium oxide were 10 and 30 g, respectively. A–D: the volume of water was 4, 5, 6, and 7 mL, respectively. Data shown as mean $\pm$ SD, $n = 3$.

was not solved. Thus, to further prove the capacity for detection, our HNB detection method was introduced to realize instant visualization of LAMP products without specialized equipment, and the LAMP assay could not be terminated. Meanwhile, the process of IMS, DNA extraction, and LAMP-HNB assay was performed using an electricity-free heater and conventional electric device, respectively. Then, the LAMP products obtained by two devices were observed by AGE under UV light (Figure 5A) and color of HNB (Figure 5B). The results of AGE demonstrated that an electricity-free heater can supply the same reaction conditions as a conventional electric heater, and the LAMP reaction in a portable electricity-free heater produced a pattern of electrophoresis similar to that of a conventional electric heater. Meanwhile, the results of LAMP products by HNB method (via color changing) were in accordance with the AGE assay, which further illustrated the favorable stability of the electricity-free heater. At the same

time, the ratio of OD_{500nm} and OD_{650nm} were tested to verify the rationality of the visual detection method (Supplemental Table S1; <https://doi.org/10.3168/jds.2019-17661>). The results also showed a significant difference between positive samples from 2 devices and negative control. Thus, the feasibility of the visual detection system was confirmed by detecting the *Cronobacter* spp., and the total detection process did not require expensive instruments and skilled operators. Furthermore, the total visual detection time containing IMS, DNA extraction, and LAMP-HNB assay can be controlled in 2 h 30 min without preincubation steps of bacteria.

Specificity of LAMP Assay with a Portable Electricity-Free Heater

Our heater as heat source needed warm-up at lower reaction temperature to implement the LAMP assay

Figure 5. The results of detection of *Cronobacter* spp. by conducting immunomagnetic separation, DNA extraction, and loop-mediated isothermal amplification in the different devices. (A) Agarose gel electrophoresis. (B) Hydroxynaphthol blue. M: marker; lane 1: portable electricity-free heater; lane 2: conventional electric heater; N: negative control.

and the LAMP primers were designed in a previous screening. To test if the LAMP assay with the electricity-free heater could produce specific amplification and specificity of LAMP primers, the LAMP products were observed by changing color of HNB and AGE under UV light, respectively. The results demonstrated that all 19 *Cronobacter* spp. strains showed a sky blue color and all 25 non-*Cronobacter* spp. strains were violet by the HNB method with a portable electricity-free heater as shown in Table 1. Meanwhile, the positive reaction was seen as a ladder-like pattern on 2% AGE analysis, which contained all 19 *Cronobacter* spp. strains, whereas no band of the negative reaction contained all 25 non-*Cronobacter* spp. strains.

The sequences of positive amplified products were measured by utilizing DNA sequencing technology, which illustrating the consistency with targeted fragments in ITS. At the same time, LAMP primers were proven to be specific to the selected region and used to identify *Cronobacter* spp. with our electricity-free amplification detection system. Similar results appeared in previous reports. Liu et al. (2012) used ITS sequence of *Cronobacter* spp. to design LAMP primers for rapidly detecting *Cronobacter* spp., and no false-positive amplification was observed in the 14 non-*Cronobacter* spp. microorganisms. Fu et al. (2018) developed a LAMP-LFD method based on the ITS sequence for visual detection of *Cronobacter* spp., which showed positive results of 19 *Cronobacter* spp. strains and negative results of all 29 strains of non-*Cronobacter*. These further illustrated the reliability of the 16S–23S ITS sequence to distinguish *Cronobacter* spp. from all other bacteria.

Sensitivity for *Cronobacter* spp. in Artificially Contaminated PIF

To test the sensitivity of our electricity-free amplification and visual detection system, *C. sakazakii* ATCC 29544 was chosen as the target bacterium for performing IMS, DNA extraction, and LAMP assay. Meanwhile, the sensitivity was determined to compare with a conventional electric device and traditional PCR method by amplifying target bacteria in PIF at known concentrations. In this paper, the sensitivities for PIF contaminated with *C. sakazakii* using a conventional electric device and electricity-free heater manifested as shown in Figure 6. The results of HNB and AGE illustrated that the detection limit of the contaminated *Cronobacter* spp. in PIF was 3.8×10^2 cfu/g in electric devices by using 2 detection methods. The detection limit was 4.2×10^2 cfu/g in an electricity-free heater, whereas it was 2.6×10^3 cfu/g with the PCR method, indicating no significant difference by utilizing an electricity-free heater and conventional electric devices, and the LAMP-HNB method established in this investigation had the same sensitivity as the LAMP-AGE, which was 10 times that of the traditional PCR method. In previous reports, Chen et al. (2017) developed the IMS-PCR method to detect *Cronobacter* spp. and the sensitivity was 5.2×10^2 cfu/mL in artificially contaminated PIF without enrichment. The detection limit in milk was 2.8×10^4 cfu/mL by utilizing a noninstrumented platform (Kubota et al., 2013). Therefore, the established system was certified to have high sensitivity. To further realize the applicability for real samples, we found the established system could detect *Cronobacter* spp. with a level of 10^0 cfu/g in artificially contaminated PIF after 2 h of cultivation (Supplemental Table S2; <https://doi.org/10.3168/jds.2019-17661>).

Interference Test for LAMP Using Various Concentrations of Non-*Cronobacter* spp.

There generally existed background microflora in PIF in addition to the *Cronobacter* spp., which could affect the LAMP reaction to cause false-positive or false-negative results. Hence, a mixture of DNA from *C. sakazakii* and 4 non-*Cronobacter* spp. in PIF was used to evaluate LAMP interference by our electricity-free heater. As shown in Figure 7, the sky blue color of HNB and trapezoidal bands of AGE appeared with bacterial concentrations from 10^2 to 10^8 cfu/g, whereas the violet color and no bands appeared when the bacterial concentrations were less than 10^2 cfu/g. The results were consistent with the specificity and sensitivity detection of *Cronobacter* spp., indicating that our electricity-free

Figure 6. The sensitivity for powdered infant formula (PIF) contaminated with *Cronobacter* spp. using different devices and methods. (A) Conventional electric heater. Lanes 1 to 6: the PIF contaminated with concentrations of *Cronobacter sakazakii* ATCC 29544 ranging from 3.8×10^4 to 3.8×10^{-1} cfu/g, respectively. (B) Electricity-free heater. Lanes 1 to 6: the PIF contaminated with concentrations of *C. sakazakii* ATCC 29544 ranging from 4.2×10^4 to 4.2×10^{-1} cfu/g, respectively. Loop-mediated isothermal amplification products were examined by hydroxynaphthol blue (upper lane) and agarose gel electrophoresis (lower lane). (C) Traditional PCR method. Lanes 1 to 6: the PIF contaminated with concentrations of *C. sakazakii* ATCC 29544 ranging from 2.6×10^4 to 2.6×10^{-1} cfu/g, respectively. M: marker; N: negative control.

amplification and visual detection system was not affected by DNA from other bacteria.

Stability Assay in Different Ambient Temperatures

Because the detection process was conducted in limited-resource settings, the equipment may suffer from high or low temperatures. Therefore, the stability of an electricity-free heater may have a fluctuant result of

unstable sensitivity of PIF contaminated with *Cronobacter* spp. To certify the stability and the reliability of detection limit of the electricity-free heater, the experiment was carried out at 3 representative temperatures of 4°C, 25°C and 37°C by using an electricity-free heater, and the products were detected by HNB. The results suggested that a stable reaction temperature could be maintained, whereas the sensitivity had no significant difference and could attain 10^2 cfu/g (Table 2). Consequently, the stability and sensitivity were reliable in ambient temperatures of operation ranging from 4°C to 37°C.

CONCLUSIONS

We designed a portable electricity-free heater that integrated the processes of IMS, DNA extraction, and LAMP assay of PIF contaminated with *Cronobacter* spp. It is a simple, low cost, chemically heated, and portable device without electrical power. At the same time, HNB was added to the LAMP reaction in advance to achieve visual detection of amplified products and avoid cross-contamination, high cost, and utilization of electrical instruments. The visual detection system we established showed high specificity for *Cronobacter*

Figure 7. Interference test for *Cronobacter* spp. with different concentrations of non-*Cronobacter* spp. M: marker; N: negative control; lanes 1–9: the powdered infant formula contaminated with *Cronobacter sakazakii* ATCC 29544 (concentration fixed at 10^7 cfu/g) and 4 non-*Cronobacter* spp. ranging from 10^0 to 10^8 cfu/g, respectively. Loop-mediated isothermal amplification products were examined by hydroxynaphthol blue (upper lane) and agarose gel electrophoresis (lower lane).

Table 2. The detection limit of the *Cronobacter* spp. in powdered infant formula using a portable electricity-free heater at different ambient temperatures

Item	Ambient temperature (°C)		
	4	25	37
Detection limit (cfu/g)	3.6×10^2	4.5×10^2	4.7×10^2

spp. and the sensitivity can reach 10^2 cfu/g in PIF, even at different ambient temperatures. The total detection time was less than 2 h 30 min. Furthermore, *Cronobacter* spp. can be detected at a concentration of 10^0 cfu/g in artificially contaminated PIF after 2 h of pre-enrichment. In short, compared with conventional LAMP assays for *Cronobacter* spp., this LAMP-HNB visual method with a portable electricity-free heater will provide a rapid, simple, specific, and sensitive detection system for *Cronobacter* spp. in PIF in limited-resource settings, and can be applied to other food products.

ACKNOWLEDGMENTS

This study was supported by National Natural Science Foundation of China (no. 31871828), National High-Level Talents Special Support Program of China, and National Key Research and Development Program of China (no. 2018YFE0120500). We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

REFERENCES

- Abdelhai, M. H., H. A. M. Hassanin, and X. Sun. 2016. Comparative study of rapid DNA extraction methods of pathogenic bacteria. *J. Biosci. Bioeng.* 4:1–8. <https://doi.org/10.11648/j.bio.20160401.11>.
- Bletz, M. C., E. A. Rebollar, and R. N. Harris. 2015. Differential efficiency among DNA extraction methods influences detection of the amphibian pathogen *Batrachochytrium dendrobatidis*. *Dis. Aquat. Organ.* 113:1–8. <https://doi.org/10.3354/dao02822>.
- Chen, Q., Y. Li, T. Tao, X. Bie, F. Lu, and Z. Lu. 2017. Development and application of a sensitive, rapid, and reliable immunomagnetic separation-PCR detection method for *Cronobacter* spp. *J. Dairy Sci.* 100:961–969. <https://doi.org/10.3168/jds.2016-11087>.
- Curtis, K. A., D. L. Rudolph, I. Nejad, J. Singleton, A. Beddoe, B. Weigl, P. Labarre, and S. M. Owen. 2012. Isothermal amplification using a chemical heating device for point-of-care detection of HIV-1. *PLoS One* 7:e31432. <https://doi.org/10.1371/journal.pone.0031432>.
- Fu, S., Y. Jiang, X. Jiang, Y. Zhao, S. Chen, X. Yang, and C. Man. 2018. Probe-free label system for rapid detection of *Cronobacter* genus in powdered infant formula. *AMB Express* 8:155. <https://doi.org/10.1186/s13568-018-0689-x>.
- Hatano, B., T. Maki, T. Obara, H. Fukumoto, K. Hagiwara, Y. Matsushita, A. Okutani, B. Bazartseren, S. Inoue, and T. Sata. 2010. LAMP using a disposable pocket warmer for anthrax detection, a highly mobile and reliable method for anti-bioterrorism. *Jpn. J. Infect. Dis.* 63:36–40. <https://doi.org/10.1258/ijisa.2009.008500>.
- Healy, B., S. Cooney, S. O'Brien, C. Iversen, P. Whyte, J. Nally, J. J. Callanan, and S. Fanning. 2010. *Cronobacter* (*Enterobacter sakazakii*): An opportunistic foodborne pathogen. *Foodborne Pathog. Dis.* 7:339–350. <https://doi.org/10.1089/fpd.2009.0379>.
- Huang, X., Z. P. Aguilar, H. Xu, W. Lai, and Y. Xiong. 2016. Membrane-based lateral flow immunochromatographic strip with nanoparticles as reporters for detection: A review. *Biosens. Bioelectron.* 75:166–180. <https://doi.org/10.1016/j.bios.2015.08.032>.
- Kim, H. S., Y. J. Kim, J. W. Chon, D. H. Kim, and K. H. Seo. 2016. Two-stage label-free aptasensing platform for rapid detection of *Cronobacter sakazakii* in powdered infant formula. *Sensor. Actuat. Biol. Chem.* 239:94–99. <https://doi.org/10.1016/j.snb.2016.07.173>.
- Kubota, R., P. Labarre, J. Singleton, A. Beddoe, B. H. Weigl, A. M. Alvarez, and D. M. Jenkins. 2011. Non-instrumented nucleic acid amplification (NINA) for rapid detection of *Ralstonia solanacearum* Race 3 biovar 2. *Biol. Eng. Trans.* 4:69–80. <https://doi.org/10.13031/2013.38508>.
- Kubota, R., P. Labarre, B. H. Weigl, Y. Li, P. Haydock, and D. M. Jenkins. 2013. Molecular diagnostics in a teacup: Non-instrumented nucleic acid amplification (NINA) for rapid, low cost detection of *Salmonella enterica*. *Chin. Sci. Bull.* 58:1162–1168. <https://doi.org/10.1007/s11434-012-5634-9>.
- Labarre, P., J. Gerlach, J. Wilmoth, A. Beddoe, J. Singleton, and B. Weigl. 2010. Non-instrumented nucleic acid amplification (NINA): Instrument-free molecular malaria diagnostics for low-resource settings. *Conf. Proc. IEEE Eng. Med. Biol. Soc.* 2010:1097–1099. <https://doi.org/10.1109/IEMBS.2010.5627346>.
- Labarre, P., K. R. Hawkins, J. Gerlach, J. Wilmoth, A. Beddoe, J. Singleton, D. Boyle, and B. Weigl. 2011. A simple, inexpensive device for nucleic acid amplification without electricity-toward instrument-free molecular diagnostics in low-resource settings. *PLoS One* 6:e19738. <https://doi.org/10.1371/journal.pone.0019738>.
- Liao, S. C., J. Peng, M. G. Mauk, S. Awasthi, J. Song, H. Friedman, H. H. Bau, and C. Liu. 2016. Smart Cup: A minimally-instrumented, smartphone-based point-of-care molecular diagnostic device. *Sens. Actuators B Chem.* 229:232–238. <https://doi.org/10.1016/j.snb.2016.01.073>.
- Liu, C., M. G. Mauk, R. Hart, X. Qiu, and H. H. Bau. 2011. A self-heating cartridge for molecular diagnostics. *Lab Chip* 11:2686. <https://doi.org/10.1039/c1lc20345b>.
- Liu, C., W. J. Zheng, H. W. Zhang, Y. M. Hou, and Y. Liu. 2009. Sensitive and rapid detection of *Enterobacter sakazakii* in infant formula by loop-mediated isothermal amplification method. *J. Food Saf.* 29:83–94. <https://doi.org/10.1111/j.1745-4565.2008.00142.x>.
- Liu, X., J. Fang, M. Zhang, X. Wang, W. Wang, Y. Gong, X. Xi, and M. Li. 2012. Development of a loop-mediated isothermal amplification assay for detection of *Cronobacter* spp. (*Enterobacter sakazakii*). *World J. Microbiol. Biotechnol.* 28:1013–1020. <https://doi.org/10.1007/s11274-011-0899-8>.
- Luo, K., L. Hu, Q. Guo, C. Wu, S. Wu, D. Liu, Y. Xiong, and W. Lai. 2017. Comparison of 4 label-based immunochromatographic assays for the detection of *Escherichia coli* O157:H7 in milk. *J. Dairy Sci.* 100:5176–5187. <https://doi.org/10.3168/jds.2017-12554>.
- Norberg, S., C. Stanton, R. P. Ross, C. Hill, G. F. Fitzgerald, and P. D. Cotter. 2012. *Cronobacter* spp. in powdered infant formula. *J. Food Prot.* 75:607–620. <https://doi.org/10.4315/0362-028X.JFP-11-285>.
- Pecchia, S., and D. Da Lio. 2018. Development of a rapid PCR-nucleic acid lateral flow immunoassay (PCR-NALFIA) based on rDNA *IGS* sequence analysis for the detection of *Macrophomina phaseolina* in soil. *J. Microbiol. Methods* 151:118–128. <https://doi.org/10.1016/j.mimet.2018.06.010>.
- Sema, M., A. Alemu, A. G. Bayih, S. Getie, G. Getnet, D. Guelig, R. Burton, P. Labarre, and D. R. Pillai. 2015. Evaluation of non-instrumented nucleic acid amplification by loop-mediated isothermal amplification (NINA-LAMP) for the diagnosis of malaria in Northwest Ethiopia. *Malar. J.* 14:44. <https://doi.org/10.1186/s12936-015-0559-9>.
- Singleton, J., J. L. Osborn, L. Lillis, K. Hawkins, D. Guelig, W. Price, R. Johns, K. Ebels, D. Boyle, B. Weigl, and P. LaBarre. 2014. Electricity-free amplification and detection for molecular point-of-care diagnosis of HIV-1. *PLoS One* 9:e113693. <https://doi.org/10.1371/journal.pone.0113693>.
- Song, X., S. Shukla, G. Lee, S. Park, and M. Kim. 2016. Detection of *Cronobacter* genus in powdered infant formula by enzyme-linked immunosorbent assay using anti-*Cronobacter* antibody. *Front. Microbiol.* 7:1124. <https://doi.org/10.3389/fmicb.2016.01124>.
- Sun, Y., T. L. Quyen, T. Q. Hung, W. H. Chin, A. Wolff, and D. D. Bang. 2015. A lab-on-a-chip system with integrated sample preparation and loop-mediated isothermal amplification for rapid and quantitative detection of *Salmonella* spp. in food samples. *Lab Chip* 15:1898–1904. <https://doi.org/10.1039/C4LC01459F>.

- Syedmoradi, L., M. Daneshpour, M. Alvandipour, F. A. Gomez, H. Hajghassem, and K. Omidfar. 2017. Point of care testing: The impact of nanotechnology. *Biosens. Bioelectron.* 87:373–387. <https://doi.org/10.1016/j.bios.2016.08.084>.
- Wang, C. H., K. Y. Lien, J. J. Wu, and G. B. Lee. 2011. A magnetic bead-based assay for the rapid detection of methicillin-resistant *Staphylococcus aureus* by using a microfluidic system with integrated loop-mediated isothermal amplification. *Lab Chip* 11:1521–1531. <https://doi.org/10.1039/c0lc00430h>.
- Wang, Y., H. Li, Y. Wang, L. Zhang, J. Xu, and C. Ye. 2017. Loop-mediated isothermal amplification label-based gold nanoparticles lateral flow biosensor for detection of *Enterococcus faecalis* and *Staphylococcus aureus*. *Front. Microbiol.* 8:192. <https://doi.org/10.3389/fmicb.2017.00192>.
- Wang, Y., Y. Wang, R. Lan, H. Xu, A. Ma, D. Li, H. Dai, X. Yuan, J. Xu, and C. Ye. 2015. Multiple endonuclease restriction real-time loop-mediated isothermal amplification. *J. Mol. Diagn.* 17:392–401. <https://doi.org/10.1016/j.jmoldx.2015.03.002>.
- Wu, R., X. Liu, B. Guo, F. Chen, and X. Wang. 2014. Development of double loop-mediated isothermal amplification to detect *Listeria monocytogenes* in food. *Curr. Microbiol.* 69:839–845. <https://doi.org/10.1007/s00284-014-0661-1>.
- Yan, L., J. Zhou, Y. Zheng, A. S. Gamson, B. T. Roembke, S. Nakayama, and H. O. Sintim. 2014. Isothermal amplified detection of DNA and RNA. *Mol. Biosyst.* 10:970. <https://doi.org/10.1039/c3mb70304e>.
- Yan, Q., and S. Fanning. 2015. Strategies for the identification and tracking of *Cronobacter* species: An opportunistic pathogen of concern to neonatal health. *Front. Pediatr.* 3. <https://doi.org/10.3389/fped.2015.00038>.
- Yang, Y., F. Xu, H. Xu, Z. P. Aguilar, R. Niu, Y. Yuan, J. Sun, X. You, W. Lai, Y. Xiong, C. Wan, and H. Wei. 2013. Magnetic nano-beads based separation combined with propidium monoazide treatment and multiplex PCR assay for simultaneous detection of viable *Salmonella Typhimurium*, *Escherichia coli* O157:H7 and *Listeria monocytogenes* in food products. *Food Microbiol.* 34:418–424. <https://doi.org/10.1016/j.fm.2013.01.004>.
- Yin, H. Y., T. J. Fang, and H. W. Wen. 2016. Combined multiplex loop-mediated isothermal amplification with lateral flow assay to detect *sea* and *seb* genes of enterotoxigenic *Staphylococcus aureus*. *Lett. Appl. Microbiol.* 63:16–24. <https://doi.org/10.1111/lam.12590>.
- Zhao, Y., X. Jiang, Y. Qu, R. Pan, X. Pang, Y. Jiang, and C. Man. 2017. *Salmonella* detection in powdered dairy products using a novel molecular tool. *J. Dairy Sci.* 100:3480–3496. <https://doi.org/10.3168/jds.2016-12535>.

ORCIDS

- Yujun Jiang <https://orcid.org/0000-0002-2857-2137>
 Sihan Chen <https://orcid.org/0000-0002-5249-8723>
 Wei Zhang <https://orcid.org/0000-0003-0773-7788>
 Chaoxin Man <https://orcid.org/0000-0003-2050-4639>