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Research Article

Rapid Detection of *Cronobacter* and *Salmonella* in Powdered Infant Formula and Related Matrices Using Loop-mediated Isothermal Amplification (LAMP)-Bioluminescent Assays as Compared to the Guobiao Standards (GB) Method

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ABSTRACT

Cronobacter and *Salmonella* have emerged as major pathogens of concern in Powdered Infant Formula (PIF). China's PIF industry is growing, and consumers are concerned about the potential risk of these pathogens in PIF. The PIF producers need rapid, easy to use and specific detection of *Cronobacter* and *Salmonella* for monitoring of raw materials, process environment and finished products for implementing effective control measures to prevent contamination. The objective of this study was to determine the specificity and sensitivity of LAMP assays compared to the Chinese National Food Safety (Guobiao, GB) Standards 4789.4-2016 (*Salmonella*) and GB 4789.40-2016 (*Cronobacter*) detection in raw materials (milk powder, whey powder, minerals, lutein, nucleotide, arachidonic acid powder, docasahexaenoic acid), process environment samples and finished products (neonate and infant PIF). The respective LAMP assays detected *Cronobacter* and *Salmonella* in artificially contaminated samples and the results of the LAMP assays were comparable to the respective GB method. The Probability of Detection (POD) analysis showed no significant differences (95% confidence interval) among all the samples tested for both LAMP assays and the respective GB method. The respective LAMP assays enabled rapid detection of *Cronobacter* and *Salmonella* in PIF matrices providing next-day results as compared to 3 to 5 days for the GB method.

Keywords: *Cronobacter*; *Salmonella*; LAMP; GB method; Powdered infant formula; Rapid detection

INTRODUCTION

Foodborne diseases are global public health issue caused by foodborne pathogens. According to World Health Report, about 1 in 10 people around the world are affected by food contamination resulting in about 420,000 deaths annually by foodborne diseases [1]. Consumers with damaged or weak immune systems and children, especially infants and neonates are more vulnerable to foodborne diseases from contaminated food.

Contamination of Powdered Infant Formula (PIF) with *Cronobacter* and *Salmonella* and the management of risk to consumers is a major concern to public health and regulatory officials and manufacturers [2-6]. *Salmonella* and *Cronobacter* have been linked to several outbreaks and clear evidence of causality has been established for PIF. *Cronobacter* multi-species complex (previously *Enterobacter sakazakii*) is a conditional pathogen that can affect infants causing infant meningitis, necrotizing enterocolitis, bacteremia, and neonate deaths [6]. *Salmonella* infection typically causes diarrhea and, in some infants, bacteremia and meningitis. Several serotypes of *Salmonella* (Kedougou, Derby, Tennessee, Bredeney, Ailing, Virchow, Anatum and Agona) have been linked to outbreaks with PIF [2,4,7].

The contamination of finished products can occur through raw materials and the processing environment [2,6-9]. The primary source of *Cronobacter* has been found to be PIF residues, fluid beds, drying areas, floors, and soil adjacent to the production facilities [6,8-11]. The drying tower has been identified as one of the sources of *Salmonella* [2,4,7].

Mainland China has a birth rate of about 1.14% leading to almost 16 million births/year and about 85% of the newborn are formula-fed [12]. Therefore, the microbial safety and quality of the PIF is very important for China consumers. In 2019, China had over 100 factories producing 730,000 tons of PIFs [10,11]. Current international regulations require zero tolerance for *Cronobacter* spp. and *Salmonella* spp. in PIF [4-6]. With the enactment of the 2015 Food Safety Law of the People's Republic of China, prepackaged foods including general and infant food need to comply with the quality and hygienic test requirements in the applicable Chinese National Food Safety (Guobiao, GB)

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Standards [13]. The traditional GB 4789.4-2016 (*Salmonella*) and GB 4789.40-2016 (*Cronobacter*) methods require 3 to 5 days to provide results [14,15].

While the advances in rapid methods such as immunoassays and PCR have enabled accurate detection of foodborne pathogens [16-18], there is still a need for faster and simpler technology for foodborne pathogen detection to enable PIF producers to assess risks in a timely manner. With the advance of new molecular methods, Loop-mediated Isothermal Amplification (LAMP) has emerged as an alternative method to PCR. LAMP can amplify DNA under isothermal conditions (60 to 65°C) with high specificity and sensitivity in 60 min or less [17,19,20]. The DNA amplification is driven by *Bst* polymerase, a unique enzyme with DNA strand-displacement activity that enables the continuous, rapid isothermal amplification of DNA. LAMP uses multiple primers to recognize distinct regions of the genome and *Bst* DNA polymerase to provide continuous and rapid amplification of genetic material. An extension of LAMP, LAMP-bioluminescent assay, utilizes LAMP for DNA amplification and bioluminescence for the detection of amplified products [21]. Both amplification and detection occur simultaneously and continuously during the exponential phase providing real-time results and a short run time.

The LAMP-bioluminescent assays, 3M Molecular Detection System Assay 2-*Cronobacter* and 3M Molecular Detection Assay 2-*Salmonella* have been used for detection in a variety of food matrices and environmental samples and have been shown to be equivalent to standard culture methods [22,23]. The objective of this study was to determine the specificity and sensitivity of the respective LAMP assays to detect *Salmonella* and *Cronobacter* in PIF, related raw materials and environmental samples as compared to the respective traditional GB method (GB 4789.4-2016 and GB 4789.40-2016).

MATERIALS AND METHODS

Inoculum preparation

Cronobacter sakazakii (ATCC 29544, American Type Culture Collection, Manassas, VA, USA), *Salmonella enterica* subsp. *enterica* serovar Typhimurium (ATCC 14028) and *Escherichia coli* (ATCC 25922) were used for inoculation of matrices in different experiments. The strains obtained were streaked onto nutrient agar and incubated for 24 hours at 37°C. To prepare inoculum, an isolated colony from nutrient agar plate was inoculated into 100 mL of brain heart infusion broth using a sterile inoculating loop and incubated for 24 hours at 37°C. After incubation, serial 10-fold dilutions of cultures were prepared in Butterfield's phosphate buffer (pH 6.8), plated on 3M Petrifilm Aerobic Count Plate (3M Food Safety, St. Paul, MN, USA) and incubated at 37°C for 24 hours. The colonies on the plates were counted, and an average count of each dilution was used to determine the appropriate amount of inoculum to add to each sample.

Method comparison study

In a paired study, 185 samples including raw materials (n=50), PIF (n=125), and environmental (n=10) samples were used for detection of *Cronobacter*. The samples were analyzed by the LAMP method and compared with the reference GB method for the detection of *Cronobacter* spp. For *Salmonella* detection, 74 samples including raw materials (n=20), PIF (n=44), and environmental (n=10) samples were used. Out of these samples, 14 PIF samples and 10 environmental samples had dual inoculation of *Cronobacter* and *Salmonella*. In addition, 10 PIF samples had *E. coli* as an interferent organism.

Enrichment of samples

For uninoculated samples, 100 g of the raw materials (n=21) and PIF (n=37) samples were weighed into a stomacher bag and enriched in 900 mL pre-warmed BPW ISO for 18 hours at 37°C. For inoculated samples, 100 g of the raw materials (n=29) and PIF (n=64) samples were weighed into a stomacher bag and inoculated with 100 mL. inoculum to obtain about 0.1-10 CFU/raw materials sample and 1-10 CFU/PIF sample. In separate experiments, PIF and environmental samples were inoculated with both *C. sakazakii* and *S.* Typhimurium (10 or 100 CFU of each/sample). In addition, *E. coli* (about 100 CFU) was used as an interferent with some of the samples. The PIF samples were enriched in 900 mL pre-warmed BPW ISO for 18 hours at 37°C. Environmental samples were collected from a processing facility using 3M Hydrated Sponge with neutralizing buffer (3M Food Safety). The environmental samples were enriched in 225 mL of BPW ISO for 18 hours at 37°C. After enrichment, samples were analyzed by the LAMP assays and GB method.

Cronobacter and *Salmonella* detection

The enriched samples were tested with the *Cronobacter* LAMP assay (MDA2CRO) and *Salmonella* LAMP assay (MDA2SAL) obtained from 3M Food Safety. A 20 µL of sample after enrichment was collected and processed for detection following manufacturer's instructions [22,23]. All samples were culture-confirmed following the GB 4789.40-2016 (*Cronobacter*) (Figure 1) and GB 4789.4-2016 (*Salmonella*) (Figure 2). All bacterial culture media for the GB method were obtained from Beijing Land Bridge Technology Co. Ltd., Beijing, China. Biochemical confirmation of isolated colonies was done using API 20E strips (bioMérieux China Limited, Beijing, China) [14,15].

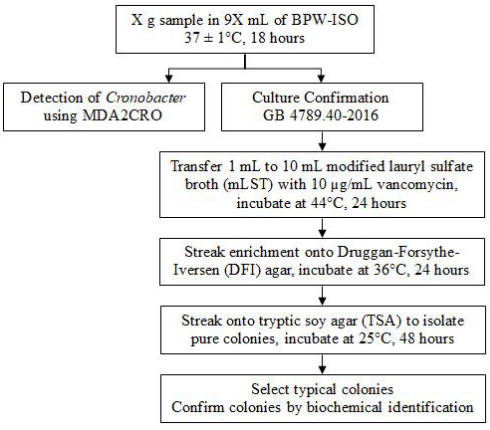

Figure 1: Paired analysis with the LAMP assay, 3M Molecular Detection Assay 2 - *Cronobacter* and the GB 4789.40-2016 method.

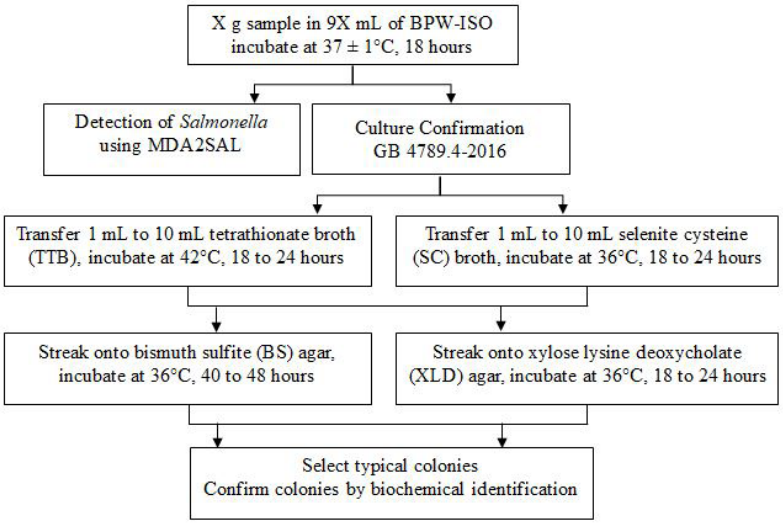

Figure 2: Paired analysis with the LAMP assay, 3M Molecular Detection Assay 2 - *Salmonella* and the GB 4789.4-2016 method.

Analysis of results

Presumptive results obtained for *Cronobacter* and *Salmonella* detection with the LAMP assays were compared with the culture-confirmed results. Probability of Detection (POD) was computed for the LAMP method (POD alternate, PODa) and the culture confirmation by GB method (POD reference, PODr) and used as a statistical model to compare the LAMP method to reference method [24]. The difference between PODa and PODr, dPOD was computed and 95% confidence interval for POD (paired analysis) was calculated. The specificity and sensitivity of each LAMP method was calculated according to ISO 16140-2: 2016 [25].

RESULTS AND DISCUSSION

Cronobacter detection

For the raw material samples, two samples out of 21 uninoculated and 9 samples out of 29 inoculated were positive by the LAMP assay. For the PIF samples, three samples out of 37 uninoculated and 62 samples out of 63 inoculated samples were positive by the LAMP assay. The presumptive positives (two from each of uninoculated raw materials and PIF samples and one from each of inoculated raw material and PIF samples) were not confirmed by the GB culture method. With dual inoculated PIF and environmental samples, presumptive results from the LAMP assay for *Cronobacter* were in complete agreement with the GB culture method. For dual inoculated PIF samples with *E. coli* as an interferent organism, three presumptive positive samples could not be confirmed by the culture method. The specificity and sensitivity of the *Cronobacter* LAMP assay for the matrices tested was 91% and 100%, respectively. The raw material samples inoculated with *Cronobacter* had low positive rate (31%) compared to PIF (96%). This could be due to the inhibitory effect of raw materials on growth of *Cronobacter* (Table 1).

Cronobacter LAMP assay had few false-positives (9 out of 185 samples) with the samples tested as some of the presumptive positive results could not be confirmed by the culture method. The LAMP assay had no false-negatives with any of the samples tested. It is possible that the samples had non-viable cells or free DNA from dead cells, and this may have contributed to false-positive results. Molecular methods including LAMP are not able to distinguish non-viable from viable cells leading to false-positive results [17,18,26]. PIF are not necessarily sterile and spray-drying used does not act as a kill step [6,8,9,27-29]. *Cronobacter* can survive the drying process and the cells may be damaged and not culturable. Also, nucleic acids are relatively stable even after cell death and may be present in food matrices after heat treatment [6,29]. Hence, it is possible that the samples had free DNA or non-culturable cells leading to false-positive results. Methods have been developed using DNase I treatment before extraction of DNA from viable cells to prevent false-positive results [30]. DNase I treatment was not evaluated in this study.

Salmonella detection

Two samples out of 20 inoculated raw material samples and all 20 inoculated PIF samples were positive by the LAMP assay and all the presumptives results were confirmed by the GB culture method. With dual inoculated PIF and environmental samples and PIF samples with *E. coli* as an interferent organism, presumptive results from the *Salmonella* LAMP assay were in complete agreement with the GB culture method. Both specificity and sensitivity of the *Salmonella* LAMP assay was 100%. The raw material samples inoculated with *Salmonella* had low positive rate (10%) compared to PIF (100%). This could be due to the inhibitory effect of raw materials on growth of *Salmonella*. (Table 2)

Data analysis

Analysis of dPOD for PIF, raw materials and environmental samples showed that the detection of *Cronobacter* spp. with the *Cronobacter* LAMP assay was not significantly different from the GB 4789.40-2016 reference method (Table 3). Similarly, the detection of *Salmonella* in PIF, raw materials and environmental samples by the *Salmonella* LAMP assay was not significantly different from the GB reference method (Table 4).

Table 1: Paired comparison between the LAMP assay and the GB method for the detection of *Cronobacter* in PIF and related matrices.

Sample[a,b]	N	MDA2CRO (Presumptive)[c]	GB method (confirmed)[d]
Cronobacter			
Raw materials uninoculated (100 g sample)	21	2	0
Raw materials with *Cronobacter* (about 0.1-10 CFU/100 g sample)	29	9	8
PIF Uninoculated (100 g sample)	37	3	1
PIF with *Cronobacter* (about 1-10 CFU/100 g sample) *Cronobacter* plus *Salmonella*	64	62	61
PIF with *Cronobacter* and *Salmonella* (about 1-10 CFU/100 g sample for each strain)	14	14	14
PIF with *Cronobacter* and *Salmonella* (about 10 or 100 CFU of each) and *E. coli* (about 100 CFU) per 100 g sample	10	10	7
Environmental samples with *Cronobacter* and *Salmonella* (about 10 or100 CFU of each) and *E. coli* (about 100 CFU)	10	7	7

[a]Samples included neonate and infant powdered infant formula, raw materials (milk powder, whey powder, minerals, lutein, nucleotide, arachidonic acid powder, docasahexaenoic acid).

[b]One-hundred gram samples (PIF and raw materials) were enriched in 900 mL of BPW ISO and incubated at 37°C for 18 hours. Environmental sponges were enriched in 225 mL BPW ISO and incubated at 37°C for 18 hours.

[c]Number of positive samples detected by the LAMP assay, MDA2CRO.

[d]Number of samples detected through culture. All samples were culturally confirmed regardless of presumptive results using GB 4789.40-2016 method [15]

Table 2: Paired comparison between the LAMP assays and the GB method for the detection of *Salmonella* in PIF and related matrices.

Sample[a]	N	MDA2SAL (Presumptive)[b]	GB method (confirmed)[c]
Salmonella[d]			
Raw materials with *Salmonella* (about 0.1-1 CFU/25 g sample)	20	2	2
PIF with *Salmonella* (about 0.1-10 CFU/25 g sample) *Cronobacter* plus *Salmonella*	20	20	20
PIF with *Cronobacter* and *Salmonella* (about 1-10 CFU/100 g sample for each strain)[e]	14	14	14
PIF with *Cronobacter* and *Salmonella* (about 10 or 100 CFU of each) and *E. coli* (about 100 CFU) per 100 g sample[e]	10	7	7
Environmental samples with *Cronobacter* and *Salmonella* (about 10 or 100 CFU of each) and *E. coli* (about 100 CFU)[e]	10	7	7

[a]Samples included neonate and infant powdered infant formula, raw materials (milk powder, whey powder, minerals, lutein, nucleotide, arachidonic acid powder, docasahexaenoic acid)

[b]Number of positive samples detected by the LAMP assay, MDA2SAL

[c]Number of samples detected through culture. All samples were culturally confirmed regardless of presumptive results using GB 4789.4-2016 method.

[d]Twenty-five gram samples were enriched in 225 mL of BPW ISO and incubated at 37°C for 18 hours.

[e]One-hundred gram samples (PIF and raw materials) were enriched in 900 mL of BPW ISO and incubated at 37°C for 18 hours. Environmental sponges were enriched in 225 mL BPW ISO and incubated at 37°C for 18 hours [14].

Table 3: POD analysis for paired comparison of *Cronobacter* detection in PIF and related matrices.

Sample	N[a]	MDA2CRO (Presumptive)	POD_a[b]	GB method (confirmed)	POD_r[c]	dPOD[d]	95% CI[e]	
							LCL	UCL
Cronobacter								
Raw materials uninoculated (100 g sample)	21	2	0.1	0	0	0.1	-0.08	0.27
Raw materials with *Cronobacter* (about 0.1-10 CFU/100 g sample)	29	9	0.31	8	0.28	0.03	-0.08	0.15
PIF Uninoculated (100 g sample)	37	3	0.08	1	0.03	0.05	-0.05	0.16
PIF with *Cronobacter* (about 1-10 CFU/100 g sample)	64	62	0.97	61	0.95	0.02	-0.04	0.07
Cronobacter plus *Salmonella*								
PIF with *Cronobacter* and *Salmonella* (about 1-10 CFU/100 g sample for each strain)	14	14	1	14	1	0	-0.19	0.19
PIF with *Cronobacter* and *Salmonella* (about 10 or 100 CFU of each) and *E. coli* (about 100 CFU) per 100 g sample	10	10	1	7	0.7	0.3	-0.07	0.67

[a]N: Total number of samples analyzed.

[b]PODa: Probability of Detection for the alternative LAMP assay.

[c]PODr: Probability of Detection for the GB 4789.40-2016 culture reference method.

[d]dPOD: Differential between the PODa and the PODr.

[e]95% CI: LCL is the lower confidence level, UCL is the upper confidence level. If the confidence interval (CI) of a dPOD contains zero, then the difference is not statistically significant at the 5% level.

Table 4: POD analysis for paired comparison of *Salmonella* detection in PIF and related matrices.

Sample	N[a]	MDA2SAL (Presumptive)	POD[b] a	GB method (confirmed)	POD[c] r	dPOD[d]	95% CI[e]	
							LCL	UCL
Salmonella								
Raw materials with *Salmonella* (about 0.1-10 CFU/25 g sample)	20	2	0.1	2	0.1	0	-0.13	0.13
PIF with *Salmonella* (about 1-10 CFU/25 g sample)	20	20	1	20	1	0	-0.13	0.13
Cronobacter plus *Salmonella*								
PIF with *Cronobacter* and *Salmonella* (about 1-10 CFU/100 g sample for each strain)	14	14	1	14	1	0	-0.19	0.19
PIF with *Cronobacter* and *Salmonella* (about 10 or 100 CFU of each) and *E. coli* (about 100 CFU) per 100 g sample	10	7	0.7	7	0.7	0	-0.25	0.25
Environmental samples with *Cronobacter* and *Salmonella* (about 10 or 100 CFU of each) and *E. coli* (about 100 CFU)	10	7	0.7	7	0.7	0	-0.25	0.25

[a]N: Total number of samples analyzed.

[b]PODa: Probability of Detection for the alternative LAMP assay.

[c]PODr: Probability of Detection for the GB 4789.4-2016 culture reference method.

[d]dPOD: Differential between the PODa and the PODr.

[e]95% CI: LCL is the lower confidence level, UCL is the upper confidence level. If the confidence interval (CI) of a dPOD contains zero, then the difference is not statistically significant at the 5% level.

LAMP is recognized throughout the scientific literature as a highly robust, efficient, sensitive, specific, and simple nucleic acid amplification technique [17,19,20]. LAMP uses a unique DNA polymerase for continuous DNA amplification that is resistant to matrix interference and inhibitors [17,19,20,31,32]. LAMP assays have the same or higher sensitivity compared to PCR assays and traditional culture methods in detecting foodborne pathogens, such as *Salmonella* spp., *Campylobacter* spp., *Listeria* spp., and *Listeria monocytogenes*, from various food matrices [17,22,23,31-34]. There have been limited studies on the comparison of rapid detection methods to GB standard methods. China's PIF industry is growing, and consumers are concerned about the potential risk of these pathogens in PIF. The PIF producers need rapid, easy to use and specific detection of *Cronobacter* and *Salmonella* for monitoring of raw materials, process environment and finished products for implementing effective control measures to prevent contamination.

In addition, interference has been observed, especially for *Cronobacter* on typical agars used for isolation. Bacteria, like *Franconibacter* spp. and *Siccibacter* spp. show typical *Cronobacter* phenotype on chromogenic agar and need further biochemical confirmation [35,36]. While colony confirmation is still relevant to laboratory testing, it is also important to recognize the higher specificity of molecular detection methods for pathogen testing which allow next-day results as compared to 3 to 5 days for traditional testing [16-18].

CONCLUSION

This study compared the LAMP assays against the GB method for detection of *Cronobacter* and *Salmonella* in PIF and related matrices. The study also evaluated the detection of both target organisms in the same enrichment and the LAMP assays detected both organisms equally well without any interference. The results of LAMP assays were similar to the GB method and provided next-day results compared to the GB method requiring 3 to 5 days.

DNA-based assays target specific genes of the target bacterium offering sensitive and specific detection. The LAMP assays used in this study offered an easy-to-use analytical tool to assess the prevalence of *Cronobacter* and *Salmonella* in PIF, raw material and environmental samples.

AUTHOR'S CONTRIBUTION

Conceptualization, Yan Huang, Jianwei Huo, Zhiyong Dai, Chenyan Niu, Xiqing Wang, Can Yi, Jichao Liu, Jun Zhou, Feng Liu, Qing Tao; methodology, Jianwei Huo, Yan Huang, and Raj Rajagopal; data generation, Jianwei Huo, Yan Huang, Qing Tao; data analysis, Yan Huang, Raj Rajagopal; Writing-original draft, Raj Rajagopal; writing - review and editing, Yan Huang and Raj Rajagopal. All authors have read and agreed to the submitted version of the manuscript.

CONFLICT OF INTEREST

The authors, Jianwei Huo, Yan Huang, and Raj Rajagopal are employees of 3M Food Safety which offers multiple commercial solutions, including 3M Molecular Detection system and 3M Molecular Detection Assays to the food industry. Reference to any commercial materials, equipment, or process does not in any way constitute approval, endorsement, or recommendation by the Ausnutria Dairy (China) Co., Ltd., Beijing Sanyuan Foods Co., Ltd., and Synutra Nutritional Food Co., Ltd.

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