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Rapid and sensitive detection of *Enterobacter sakazakii* by cross-priming amplification combined with immuno-blotting analysis

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ABSTRACT

Enterobacter sakazakii is a widespread and life-threatening bacterium especially in polluted powdered infant milk formula. Several methods have been developed for detection of *E. sakazakii* such as physiological and biochemical methods, PCR and loop-mediated isothermal amplification. However, these procedures were disadvantages due to a long assay time, low sensitivity or the use of toxic reagents. Our method of cross-priming amplification (CPA) under isothermal conditions combined with immuno-blotting analysis made the whole detection procedure more sensitivity and lower time-consuming. A set of specific displacement primers, cross primers and testing primers were designed based on six specific sequences in *E. sakazakii* 16S-23S rDNA internal transcribed spacer. Under isothermal condition at 63 °C for 60 min, the specific amplification and hybridization steps were processed simultaneously. The specificity of the CPA was tested in panel of 54 different bacterial strains and 236 milk powder products. Two red signal lines were developed on the BioHelix Express strip in all of positive *E. sakazakii* strains, and only one signal line was demonstrated by non- *E. sakazakii* bacterial strains. The limit of decetion of CPA was 6.3 ± 2.7277 fg for the genomic DNA, 88 ± 8.7892 cfu/ml for pure bacterial culture, and 3.2 ± 2.0569 cfu per 100 g milk powder with pre-enrichment. The current study demonstrated that the assay method of CPA combined with immuno-blotting analysis was a specific and sensitive detection for the rapid detection of *E. sakazakii*.

1. Introduction

Enterobacter sakazakii, which was known as "yellow pigmented *Enterobacter cloacae*", was classified as a unique species by Farmer in 1980, based on molecular differences and biochemical detection [1]. *E. sakazakii* is an opportunistic pathogen that can cause life-threatening disease of neonatal meningitis, bacteraemia, necrotizing enterocolitis (NEC) and sepsis [2,3], with mortality rates of 40–80% [4,5]. It reported that the rates of low-birth weight or immuno-compromised infants would be increased after infected by this bacterium [6]. Additionally, it was found that *E. sakazakii* could be isolated from almost all the environment and from a diverse range of foods [7–11], especially powdered infant milk formula (PIF) [12,13], which indicated that *E. sakazakii* is a widespread bacteria.

Several methods have been reported for the identification of *E. sakazakii*. The traditional physiological and biochemical procedures recommended by the U. S. Food and Drug Administration (USFDA) are laborious and poor efficiency (at least 7 days for final results). With the introduction of the molecular biology, a polymerase chain reaction (PCR) detection method has been developed [14]. But this protocol was restricted by the laboratory apparatus and also time-consuming by taking at least 3 h. A loop-mediated isothermal amplification (LAMP) method has been developed by Liu et al. [15], which detected *E. sakazakii* without asking for the specific equipment and could finish the examination in 2 h. However, the weak point of this method was the analysis and confirmation step. The products of LAMP were always analyzed by agarose gel electrophoresis and restricted endonuclease digestion. It would take a long time and have to use toxic reagent (ethidium bromide) stain the gel.

Cross-priming amplification (CPA) is a novel technique and its mechanism was described by Fang et al. [16]. Using six to eight primers, the sensitivity and specificity of the detection procedure is increased under isothermal conditions within 1 h. Besides the advantage of sensitivity and efficiency, the highlight of the method

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is the simple analytic procedure. It requires only a water bath without toxic reagent and just takes five to ten minutes to get the final results with an immuno-blotting assay by BioHelix Express strip (BESt). In CPA assay, two testing primers are involved in the isothermal amplification and labeled with biotin and FITC, respectively. As a positive test, the end of the amplicon labeled with biotin binds the colloidal gold, and the other end labeled with FITC hybridizes anti-FITC antibody which had been located on the test line of the strip, resulting in the red colloidal gold accumulated at the test line in 5–10 min. The purpose of this study was to develop a new cross-priming amplification method for *E. sakazakii* in the food sample, based on 16S–23S rDNA internal transcribed spacer (ITS).

2. Materials and methods

2.1. Bacterial strains and genomic DNA extraction

In this study, 22 *E. sakazakii* strains, with 4 ATCC and 18 strains isolated from food and human, and 32 other bacteria were tested (Table 1). All of the food and clinical samples were identified by Tianjin Entry-Exit Inspection and Quarantine Bureau, P. R. China (CIQ Tianjin), according to the USFDA standard methods. Bacterial genomic DNA was extracted from each strain by Wizard Genomic DNA Purification Kit (Promega, Inc., Madison, USA), and stored at −20 °C for use.

2.2. PCR reaction conditions

PCR reaction was performed in a total 25 μl reaction system containing 12.5 μl of 2×PCR Master Mix (0.05 units/μl *Taq* DNA polymerase, 4 mM $MgCl_2$, 0.4 mM of each dNTP) (Fermentas), 0.5 μM each primer (forward primer 5′- GGGTTGTCTGCGAAAGC-GAA -3′ and reverse primer 5′- GTCTTCGTGCTGCGAGTTTG -3′) and 1 μl of certain concentration DNA template. After a 5-min denaturation at 95 °C, the PCR mixtures were subjected to 35 cycles of amplification at 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s, and final amplification at 72 °C for 10 min. The PCR products were electrophoresis on 1.5% agarose gel.

2.3. Primers for cross-priming amplification (CPA)

The primers for CPA were listed on Table 2.

2.4. CPA reaction conditions

CPA reaction was carried out in a total 20-μl reaction mixture containing 0.4 μM each of CF and CR, 0.8 μM each of DFs and DRa, 0.1 μM each of DP1s and DP2a, 0.5 mM dNTPs (Fermentas), 2 μl 10×Bst buffer, 6 units of Bst DNA polymerase large fragment (New England Biolabs), 4 mM $MgSO_4$ (Sigma), 0.5 M betaine (Sigma), and 1 μl appropriate amount of target DNA. The CPA reaction was carried out at 63 °C for 1 h and stored at 4 °C.

2.5. Analysis of CPA products

When CPA amplification finished, 6 μl product of each reaction was dipped on the BESt strip (Ustar Biotech Co., Ltd., Hangzhou, China), then the strip was taken into the assay buffer. After the CPA product was hybridized with anti-biotin and anti-FITC antibody for 10 min, the positive result demonstrated two red lines at the test and control line position, respectively. And the strip of negative reaction had only control line. The rest product was cloned and sequenced by Sangon, China.

Table 1
Bacterial strains for CPA detection.

Species	Strain	Origin
Enterobacter sakazakii	ATCC 12868	Unknown
	ATCC 29004	Unknown
	ATCC 29544	Unknown
	ATCC 51329	Unknown
	ENS 5329	Milk powder, Ukraine
	ENS 5413	Milk powder, America
	ENS 5729	Fish meal, Japan
	ENS 51024	Milk powder, India
	ENS 51107	Whey powder, Holand
	ENS 51227	Milk powder, New Zealand
	ENS 51229	Milk powder, Australia
	ENS 6607	Milk powder, Ireland
	ENS 70115	Whey powder, France
	ENS 70216	Infant milk powder, China
	ENS 70307	Whey powder, Poland
	ENS 71123	Milk powder, Canada
	Saka 100323	Whey protein powder, Argentina
	Saka 081013	Milk powder, Australia
	Saka 090318	Butter cheese, Australia
	Saka 090224	Chocolate cake mix, America
	RFS	Fish meal, Pakistan
	SHY	Puffing food, China
Enterobacter aerogenes	ATCC 13048	Unknown
	CGMCC 1.876	Unknown
	CGMCC 1.489	Unknown
Enterobacter cloacae	ATCC 13047	Unknown
	CGMCC 1.81	Unknown
	CGMCC 1.1733	Unknown
	CMCC 45301	Unknown
Enterobacter intermedium	E.int 51231	Milk powder, China
Yersinia enterocolitica	ATCC 51871	Unknown
	ATCC 9610	Unknown
	CMCC 52204	Unknown
Yersinia pseudotuberculos	ATCC 4284	Unknown
Yersinia pestis	CMCC 52001	Unknown
Salmonella typhi	ATCC 14028	Unknown
Escherichia col	ATCC 25922	Unknown
Shigella dysenteriae	CMCC 51630	Unknown
Shigella flexneri	ATCC 12022	Unknown
Shigella sonnei	ATCC 29930	Unknown
Serratia marcescens	ATCC 8100	Unknown
Klebsiella oxytoca	ATCC 13182	Unknown
Klebsiella pneumoniae	ATCC 13883	Unknown
Hafnia alvei	ATCC 29927	Unknown
Citrobacter freundii	ATCC 8090	Unknown
Proteus mirabilis	ATCC 25933	Unknown
Proteus vulgaris	ATCC 13315	Unknown
Listeria monocytogenes	ATCC 15313	Unknown
Bacteroides fragilis	ATCC 25285	Unknown
Lactobacillus acidophilus	ATCC 4356	Unknown
Staphylococcus aureus	ATCC 43300	Unknown
*Enterococcus avium*0	ATCC 14025	Unknown
Enterococcus faecalis	ATCC 29212	Unknown
Pantoea agglomerans	ATCC 27155	Unknown

ATCC, American Type Culture Collection, Rockville, MD, USA.
CGMCC, China General Microbiological Culture Collection, Berjing, P. R. China.
CMCC, National Center For Medical Culture Collections, Beijing, P.R.China.
The others were isolated by CIQ Tianjin.

2.6. Sensitivity of CPA detection in the genomic DNA

The concentration of *E. sakazakii* genomic DNA was tested by BioSpec-mini (Shimadzu Corporation, Japan) according to the protocol of the instrument (After the self-test of the instrument, adjust the base line with the solvent according to the DNA sample at 260 nm. Then, remove the blank control, test the DNA solution under the same conditions. The concentration could be read by the instrument directly.). To study the sensitivity of CPA reaction on the DNA solution, ten-fold serial dilutions (10^0 ng–10^{-2} fg) of total genomic DNA extracted from *E. sakazakii* were subject to CPA and PCR in triplicate according to “Materials and Method” 2.2, 2.4 and

Table 2
Primers sequence and genome position for CPA detection of *E. sakazakii* (GenBank AY702093.1).

Primer	Sequences (5′—3′)
DP1s	TGCGAAAGCGAAGTCC
DP2a	GCCTCGGTTGCCTATGT
CF	TCTCTCTGACACACCGCTTTTCCTAAGGGACGCCACC (PRa TTTT PFs)
CR	CCTAAGGGACGCCACCTTTTCTCTCTGACACACCGC (PFs TTTT PRa)
Biotin-DFs	Biotin-CTGACTGTAAAGTCACGTTTGAG
FITC-DRa	FITC-CTGTTTCAATTTTCAGCTTGT

2.5. And the products of PCR and CPA were assayed by 1.5% agarose gel electrophoresis and BESt strip test, respectively.

2.7. Pre-enrichment of milk powder for detection of E. sakazakii

One hundred gram of milk powder was added to 900 ml of peptone buffered water (pH 7.2) (Oxoid Limited, Hampshire, UK) at 45 °C in Erlenmeyer flasks, which were shaken by hand until the powder was dissolved. All tests were performed in triplicate (FDA, 2002). After overnight incubation at 36 for 12 h, 200 μl of the culture from each flask was added into 5 ml of brain heart infusion (BHI) broth (Oxoid Limited, Hampshire, UK). After another overnight incubation, 1 ml of each culture was transferred to 1.5-ml microcentrifuge tube and centrifuged at 12,000 rpm for 10 min. After washing gently twice with pure water (Promega, Inc., Madison, USA), the bacterial pellet was resuspended in 50 μl TE buffer (Sangon, Shanghai, China) and boiled described as Shen et al. [17].

2.8. Sensitivity of CPA for E. sakazikii in pure culture and in commercial milk powder with enrichment

E. sakazakii was cultured in Luria-Bertani (LB) broth (10 g bacto tryptone, 5 g yeast extract and 5 g NaCl) and grown at 37 °C for 16 h to about 10^8 cfu/ml. Then the culture was ten-fold serial diluted (10^8–10^0) by saline solution in triplicate. Bacteria were counted by LB agar plate (supplemented with 15 g bacto agar per liter LB broth) in 44 °C over night. The genomic DNA of *E. sakazakii* was extracted from 1 ml of the culture by boiling the collected bacteria in 50 μl TE buffer (Sangon, Shanghai, China), described as Shen et al. [17] and 1 μl of the DNA extracted solution was used as template. To investigate CPA sensitivity in milk powder, *E. sakazakii* was serial diluted between 10^2 and 10^0 cfu and inoculated to 100 g milk powder which had been tested no contamination. This reconstituted sample was to pre-enrichment and CPA assay in triplicate.

2.9. Statistical analysis

The mean values of the sensitivity of genomic DNA, pure culture and commercial milk powder were analyzed using SPSS13.1. Datas were shown as Means ± SD.

3. Results

3.1. Primer design for CPA

A set of six primers, including two displacement, two cross, and two detector primers, which recognized six distinct regions on ITS sequence was designed by primer software Primer Premier 5.0. The primer sequences were compared to an alignment of *E. sakazakii* ITS sequence. The two displacement primers were described as displacement primer 1 sense (DP1s) and displacement primer 2 anti-sense (DP2a), The cross primers were designated as cross primer forward sense (PFs), and cross primer reverse anti-sense (PRa). Both of the cross primer forward and reverse sequences constituted cross forward primer (CF) and cross reverse primer (CR). The pair of detector primer was designated as detector forward sense (DFs), 5′ labeled biotin, and detector reverse anti-sense (DRa), 5′ labeled FITC [16]. The details of the primers were shown in Table 2.

3.2. Specificity of the CPA primers

The specificity of the CPA primers was evaluated by CPA amplification of the genomic DNA extracted from 22 *E. sakazakii* strains and 32 other bacterial strains. The CPA products of all strains were tested by BESt strip described as above. The results show that two red lines (test and control lines) on the BESt strip could be seen only in the *E. sakazakii* reaction (Fig. 1). In the reaction of non- *E. sakazakii* bacterial species, only the control line was produced (Fig. 2). This result demonstrated that the CPA primers were specific to *E. sakazakii* identification.

3.3. Sensitivity comparison of PCR and CPA in test of genomic DNA of E. sakazakii

A series of ten fold diluted concentrations (10^0 ng–10^{-2} fg) of total genomic DNA were used to be template to find out the limit of *E. sakazakii* DNA concentration of CPA and PCR at the optimal condition in triplicate, respectively. After target DNA amplification, 5 μl reaction solution was subject to assay by electrophoresis in argarose gel. The results showed that the limit of PCR was 6.3 ± 2.7277 pg ($N = 9$) (Fig. 3A). Whereas, with 6 μl CPA product dipped, the red test line always could be seen on the BESt strip. The results showed that the limit was 6.3 ± 2.7277 fg ($N = 9$) of *E. sakazakii* genomic DNA for the CPA detection (Fig. 3B).

3.4. Sensitivity of CPA in pure culture

Sensitivity of CPA reaction on *E. sakazakii* was determined by analyzing product produced from the ten-fold serial dilutions of the culture of *E. sakazakii* in triplicate. After CPA amplification, with 6 μl CPA product dipped, two red lines always could be found on the strip with the genomic DNA extracted from 88 ± 8.7892 cfu/ml bacteria ($N = 9$) (Fig. 4). And the limit level of CPA to *E. sakazakii* in pure culture was about 1.76 cfu per reaction (template 88 cfu/50).

3.5. Sensitivity of CPA detection to E. sakazakii in milk powder with enrichment

Nine milk powder samples from different country (two from Australia, one from Canada, five from China and one from France)

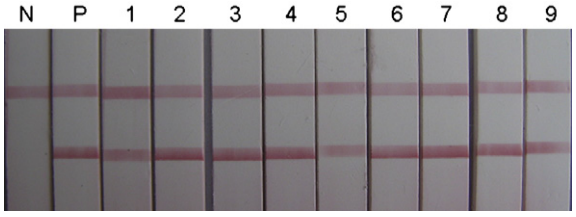

Fig. 1. CPA amplification for *E. sakazakii* strains. BESt strip detection for CPA products using genomic DNA extracted from pure cultered *E. sakazakii* (10^8 cfu/ml). Lane 1, *E. sakazakii* 12868; lane 2, *E. sakazakii* 29544; lane3, *E. sakazakii* 29004; lane 4, *E. sakazakii* 51329; lane 5, *E. sakazakii* ENS 5329; lane 6, *E. sakazakii* ENS 5413; lane 7, *E. sakazakii* ENS 5729; lane 8, *E. sakazakii* ENS 51024; lane 9, *E. sakazakii* ENS 51107; lane N, no template negative control; lane P, purified PCR product of 16S–23S rDNA ITS of *E. sakazakii* as a positive control.

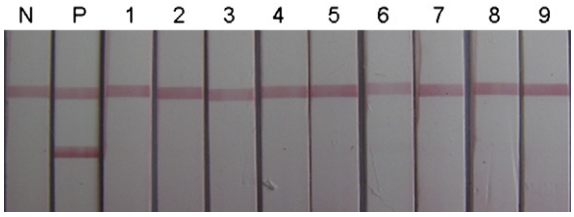

Fig. 2. CPA amplification for non-*E. sakazakii* bacterial strains. BESt strip detection for CPA products using genomic DNA extracted from pure cultered non-*E. sakazakii* strains (10^8 cfu/ml). Lane 1, *Enterobacter aerogenes*; lane 2, *Enterobacter cloacae*; lane 3, *Enterobacter intermedium*; lane 4, *Yersinia enterocolitica*; lane 5, *Yersinia pseudotuberculos*; lane 6, *Salmonella typhi*; lane 7, *Escherichia coli*; lane 8, *Shigella dysenteriae*; lane 9, *Klebsiella pneumoniae*; lane N, no template negative control; lane P, purified PCR product of 16S–23S rDNA ITS of *E. sakazakii* as a positive control.

were confirmed to be negative for *E. sakazakii*. One hundred grams of sample was inoculated in triplicate with cell dilution between 10^0 and 10^2 cfu of *E. sakazakii*. The inoculated and non-inoculated milk powder were incubated and DNA extracted as described in Materials and methods section 2.7 and 2.8. With CPA assay, the 9 inoculated samples with 3.2 ± 2.0569 cfu/100 g milk powder were tested positive, while non-inoculated samples were tested negative (Fig. 5).

3.6. Practical application of CPA detection to E. sakazakii in food samples

We employed the CPA method to the daily routine detection in Tianjin Entry-Exit Inspection and Quarantine Bureau (CIQ). To confirm the results of CPA assay the same samples had been detected by VITEK 32 system (BioMérieus, Inc., Maray l'Etoil, France) which were carried out by specialists in CIQ. In 236 milk powder products analyzed by CPA with pre-enrichment, 3 positive reactions were found by both CPA and VITEK 32 system. The quantities of *E. sakazakii* were 2.7 cfu, 5.9 cfu and 22.1 cfu/100 g milk powder respectively (Fig. 6).

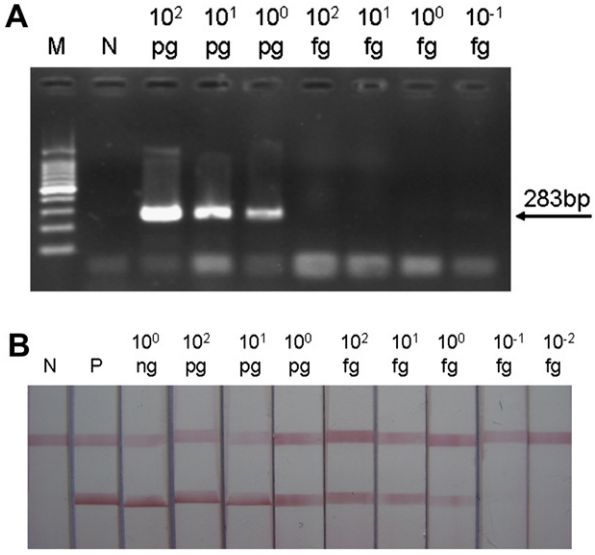

Fig. 3. Comparison of sensitivity between PCR and CPA detection for *E. sakazakii* using genomic DNA. Genomic DNA used as template was ten-fold serial dilluted between 10^0 ng and 10^{-2} fg by distilled water. (A) Agarose gel shows the limit of the PCR. (B) The immuno blot analysis of CPA products. Lane N, no template negative control; lane P, purified PCR product of 16S–23S rDNA ITS of *E. sakazakii* as a positive control.

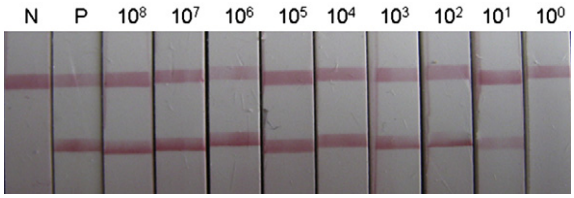

Fig. 4. Sensitivity of CPA detection for *E. sakazakii* in pure culture. The *E. sakazakii* organism (about 10^8 cfu/ml) was ten-fold serial dilluted by saline solution, and counted by LB agar plate. Lane N, no template negative control; lane P, purified PCR product of 16S–23S rDNA ITS of *E. sakazakii* as a positive control.

4. Discussion

E. sakazakii was ranked as "Severe hazard for restricted populations, life-threatening or substantial chronic sequelae or long duration" by ICMSF [18] and a category A (clear evidence of causality) pathogen bacteria [19]. Though *E. sakazakii* was identified by standard detection methods, some species of atypical observation were reported. For example, Besse et al. [20] found that some *E. sakazakii* organisms did not produce the yellow pigment on TSA, which questioned API 20E results of *E. sakazakii* identification [10,21,22].

Althrough molecular methods were common for bacterial identification, Lehner et al. [23] found that there were two phylogenetically distinct branches in the *E. sakazakii* species. The similarity of strain ATCC 51329 with type strain ATCC 29544 was significantly lower (97.9%) than that of the other *E. sakazakii* strains (99.4 to 100%). And *E. sakazakii* ATCC 51329 could not be detected by PCR identification system [24]. To resolve the problem, in our work a set of six specific primers were designed by blasting the 16S–23S rDNA ITS sequences of 13 *E. sakazakii* strains, including ATCC 29544 and 51329, for the CPA identification method.

Compared with the determination of amplification product used, the immuno-blotting analysis was more rapid and efficient, especially in field use [25]. Recently, some methods using antigen-antibody interaction were reported for virus detection such as Taura syndrome virus [26], *Penaeus monodon* densovirus [27], infectious myonecrosis virus [28], *P. monodon* nucleopolyhedrovirus [25]. All of these results indicated that the immunological detection was sensitive as gel electrophoresis. In the present study, no gel electrophoresis was used to test the amplification product, and instead the BESt strip assay could demonstrate the results directly. The limit of the CPA detection was 10^0 fg of the genomic DNA, which was 10^3 times more sensitive than the PCR. It was

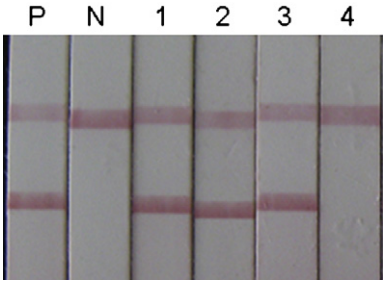

Fig. 5. Sensitivity of CPA detection to *E. sakazakii* in milk powder sample with enrichment. CPA assay results of DNA extracted from serial dilutions of milk powder artificially contaminated with *E. sakazakii* ATCC 29004 after pre-enrichment. Lane 1, 1.2×10^2; Lane 2, 1.2×10^1; Lane 3, 1.2×10^0 cfu/100 g milk powder; Lane 4, negative control of DNA extracted from milk powder without *E. sakazakii* contaminated; Lane N, no template negative control; lane P, purified PCR product of 16S–23S rDNA ITS of *E. sakazakii* as a positive control.

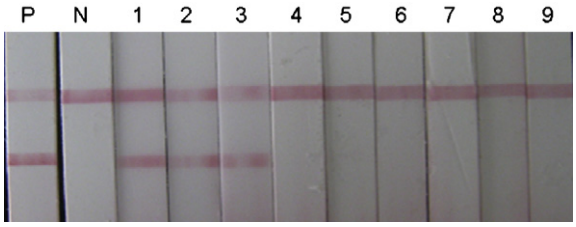

Fig. 6. CPA detection for *E. sakazakii* in daily routine milk powder products examination. Partial results of CPA method to the daily routine detection with pre-enrichment in Tianjin Entry-Exit Inspection and Quarantine Bureau (CIQ). Lane 1–3, CPA assays of positive samples with *E. sakazakii* 2.7 cfu, 5.9 cfu and 22.1 cfu/100 g milk powder, respectively; Lane 4–9, CPA assays of milk powder without *E. sakazakii* contaminated; Lane N, no template negative control; lane P, purified PCR product of 16S-23S rDNA ITS of *E. sakazakii* as a positive control.

comparative to 10^2 cfu/ml for bacteria pure culture. In daily routine detection, the CPA assay fits for the low level of 10^0 cfu *E. sakazakii* in 100 g milk powder with pre-enrichment.

The virus detection of loop-mediated isothermal amplification (LAMP) combined with immuno-blotting analysis was rapid and sensitive, but the method had a serious defect. To analyze the product by immuno blot, the FITC-labeled DNA probe need be added into the reaction system after isothermal amplification. As the hybridization temperature was the same as the amplification, the whole procedure was operated in a hot heating block or water bath. This step could threat to the health of the operators and increase the difficulty of the protocol. With the additional step and the hybridization time, the identification reaction would be extended, depending on the amount of samples.

Cross-priming amplification was a successive procedure without any pause. The labeled DNA probe in LAMP was alternated by detector primers, and the reaction mixture was prepared before the isothermal amplification. Neither additional step need be added, nor need extra time be wasted. The whole CPA detection could be finished within successive procedure of 60–70 min.

In conclusion, the detection of *E. sakazakii* by cross-priming amplification combined with immuno blot analysis is a rapid and sensitive method, especially applied in field since the system only require some simple equipments.

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