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Development of Fluorescent Microsphere-Based Immunochromatographic Strip for Rapid Detection of *Cronobacter* in Milk

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Abstract

Detection of pathogens is of great importance for health and safety. *Cronobacter* which belongs to common human opportunistic foodborne pathogen can lead to meningitis and necrotizing enterocolitis in infants. So a simple and rapid immunochromatographic strip for detecting *Cronobacter* was being more in need of and setted up for the first time in our study. CP783 protein was expressed and used as the specific antigen of *Cronobacter* to obtain polyclonal antibody (pAb) for immunochromatographic strip. Through cloning, expression, purification and animal immunization, we obtained pAb against CP783 protein, which were then conjugated to the fluorescent microsphere as the capture reagent at the test line. Meanwhile, cross reaction with *Vibrio parahaemolyticus* PVPA 0146 and *Salmonella typhimurium* ATCC 13311 determined by dot blot were eliminated after purification of pAb against *Cronobacter* whole cell protein. Afterwards, the pAb against whole cell protein was sprayed as the detective reagent. Our developed fluorescent microsphere-based immunochromatographic strip had high specificity and sensitivity. It resulted in reaching the minimum detectable concentration of *Cronobacter* at 10^5 CFU/ml in pure culture and 10^6 CFU/ml in milk.

Keywords: *Cronobacter*; CP783 protein; Fluorescent microsphere; Polyclonal antibody; Immunochromatographic strip

Introduction

Cronobacter, a gram-negative, rod-shorted facultative anaerobic bacterium, can cause meningitis and necrotizing enterocolitis in infants [1]. This emerging opportunistic food-borne pathogen is as high as 40–80% mortality rate in infected infants [2]. Old man and adult patients lacking of immunity can also infected by *Cronobacter* to cause local infection and bacteremia [3]. The World Health Organization (WHO) has announced that all *Cronobacter* species are microorganisms pathogenic for human beings [3]. According to previse reports, *Cronobacter* contamination was considered to be serious, occurring at an overall infection rate of 7.5%, with the highest level of contamination being 28.8% in China during 2010-2012 [4]. So, it is very significant to establish a rapid, specific and sensitive detection method of *Cronobacter* for preventing and controlling the food-borne disease.

Traditional method for detection of *Cronobacter* was mainly based on its colonial morphology and the characteristic of physiology and biochemistry [5]. The specimens are initially pre-enriched or enriched in a non-selective broth and then according to their biochemical and morphological characteristics. Bacteria were isolated in a selective diagnostic medium [6].This method does not need expensive experiment equipment or professional skills, but the whole process usually needs 5-7 days to complete, which is time-consuming and laborious [7]. This method also accompanies high false-positive rates [8].

In recent years, with the development of molecular biology technology, many molecular biological methods were reported. Conventional PCR [9], real-time PCR [10] and loop-mediated isothermal amplification (LAMP) assay which based on polymerase chain reaction (PCR) techniques shows high sensitive and specificity than traditional methods [11]. However, biomolecule methods need some professional, experiment equipment, such as PCR instrument and electrophoresis apparatus. The experimental process must be carried out in the professional laboratory of molecular biology. The most reagents used to analyze the PCR product are toxic, such as EB, and experimenters are frequently exposed to the ultraviolet light during the agarose gel detection processing.

Besides traditional methods and biomolecule methods, there is a method called immunochromatographic strip which

is highly sensitive and specific [12,13]. It is rapid, simple, and no time-consuming to test a sample. Besides colloidal gold nanoparticles can be labeled, many new materials also can be labeled, such as fluorescent microspheres, magnetic nanoparticles, quantum dot and so on [14]. Fluorescent microspheres as a special class of functional microspheres have stable morphology, narrow particle size, good dispersion and high luminous efficiency [15]. In recent years, many fluorescent microspheres-based immunochromatographic strips were established to detect famethazine and enrofloxacin residue which are highly sensitive and accurate [15,16]. In general, fluorescent microsphere immunoassays have shown high sensitivity, time savings, and simple operation for detection of pathogens in food [17]. We have developed a method of immunochromatographic strip to detect *Cronobacter* in milk based on fluorescent microsphere. This new method has advantages over the conventional assays or other molecular biological methods, such as easy to perform with no requirement of specialized equipment, reagent or technicians, and in rapid operation.

In this study, we report first a new immunochromatographic strip which can detect all *Cronobacter* in our laboratory. Up till now, most methods have been based on PCR reaction, just using immunochromatographic strips instead of agarose gel electrophoresis (AGE), and only few species of *Cronobacter* can be detected by Immunochromatographic strip [12,13]. Just 14 years ago, in 2002, the International Commission on Microbiological Specifications for Foods (ICMSF) finally confirmed *Cronobacter* as pathogenic bacteria to a restricted population, endangering their lives and leading to serious long-term consequences, so, there is lack of professional research on *Cronobacter* [3]. According to previous reports, the characteristics of *Cronobacter* are similar with other pathogens and the antibody of *Cronobacter* always shows cross-reaction with them, such as *Salmonella typhimurium* [18]. So, in this study, we found the specific antigen of *Cronobacter*, called CP783 protein, then the protein has been cloned and expressed in E.coli JM109 cell. After purification, the CP783 protein was used as antigen to immune mice, and then we got the specific polyclonal antibody (pAb) of *Cronobacter*. The pAb against CP783 protein was conjugated to fluorescent microsphere, and then added to the conjugate pad, and the diluted pAb against *Cronobacter* cell and goat anti-rabbit IgG were transferred onto the NC membrane. Thus, our immunochromatographic strip can detect all *Cronobacter* fast and accurately.

We estimated the specificity and sensitivity of the immunochromatographic strip with pure cultured bacteria and cultured bacteria mixed with other bacteria in milk. The results of this study showed that the bacteria minimum concentration can be detected reached 10^5 CFU/ml in pure culture and 10^6 CFU/ml in milk. Moreover, there is no cross-reaction with other bacteria.

Materials and Methods

Materials and reagents

Bacteria containing 32 *Cronobacter* were preserved in our laboratory (**Table 1**). Bovine serum albumin (BSA) and goat anti-rabbit IgG and goat anti-mice IgG antibody were purchased from the Beijing Zhongshanjinqiao Biological Technology Co., Ltd. (Beijing, China). Nitrocellulose (NC) membrane, absorbent pad, sample pad, conjugate pad, and PVC sheets were purchased from Sartorius Stedim Biotech Company (Germany). Hydrogen tetra-chloroaurate hydrate (HAuCl4) and trisodium citrate were purchased from Sigma-Aldrich Corporation (American). Approximately 0.02 M sodium phosphate buffered saline (PBS, pH 8.5) was used as incubating and washing buffer in this study. All the solvents, chemicals, and salts used in this study were of analytical grade.

Table 1: List of all strains.

Serial No.	Strain	Number
1	*Cronobacter muytjensii*	ATCC 51329
2	*Cronobacter malonatics*	CMCC 45402
3	*Cronobacter sakazakii*	ATCC 29544
4	*Cronobacter sakazakii*	YC633B
5	*Cronobacter malonatics*	JA264B
6	*Cronobacter muytjensii*	SR1074B
7	*Cronobacter sakazakii*	PESA 3
8	*Cronobacter sakazakii*	PESA 9
9	*Cronobacter sakazakii*	PESA 11
10	*Cronobacter muytjensii*	PESA 17
11	*Cronobacter sakazakii*	PESA 18
12	*Cronobacter sakazakii*	PESA 19

13	*Cronobacter spp.*	CICC 21544
14	*Cronobacter spp.*	CICC 21545
15	*Cronobacter spp.*	CICC 21550
16	*Cronobacter spp.*	CICC 21551
17	*Cronobacter spp.*	CICC 21556
18	*Cronobacter spp.*	CICC 21562
19	*Cronobacter spp.*	CICC 21564
20	*Cronobacter spp.*	CICC 21569
21	*Cronobacter spp.*	CICC 21570
22	*Cronobacter spp.*	CICC 21574
23	*Cronobacter spp.*	CICC 21589
24	*Cronobacter spp.*	CICC 21590
25	*Cronobacter spp.*	CICC 21654
26	*Cronobacter spp.*	CICC 21665
27	*Cronobacter spp.*	CICC 21674
28	*Cronobacter spp.*	CICC 22918
29	*Cronobacter spp.*	CICC 22919
30	*Cronobacter spp.*	CICC 22922
31	*Cronobacter spp.*	CICC 22923
32	*Cronobacter spp.*	CICC 22924
33	*Vibrio parahaemolyticus*	PVPA 0146
34	*Salmonella typhimurium*	ATCC 13311
35	*Listeria monocytogenesis*	CMCC 54001
36	*Candida albicans*	Z1
37	*Enteropathogenic E.coli*	CMCC 44496
38	*Esherichia coli O157:H7*	PELI 0480
39	*Shigella flexneri*	ATCC 29903
40	*Micrococcus luteus*	CMCC 28003
41	*Enterobacter cloacae*	CMCC 45301
42	*Bifidobacterium breve*	WBBR04
43	*Bifidobacterium animalis*	WBBR05
44	*Bifidobacterium bifidum*	WBBI01
45	*Bifidobacterium infantis*	WBAN07
46	*Bifidobacterium adolescentis*	WBAD08
47	*Bifidobacterium lactis*	WLABO9
48	*Bifidobacterium longum*	WBLO01
49	*Lactobacillus plantarum*	ATCC 8014
50	*Lactobacillus salivarius*	ATCC 11741
51	*Lactobacillus rhamnosus GG*	ATCC 7469

52	*Lactobacillus delbrueckii*	ATCC 9649
53	*Lactobacillus acidophilus*	ATCC 4356
54	*Streptococcus thermophilus*	WST01
55	*Lactobacillus bulgaricus*	WLAB02

Preparation of pAb against *Cronobacter*

The pAb against *Cronobacter* was provided by our laboratory and assayed by dot blot analysis. Serum antibody was purified by bacterial adsorption and saturated ammonium sulfate [$(NH_4)_2SO_4$]. The pAb can only combine bacterium of *Cronobacter* and there was no cross-reaction with others.

Preparations of pAb against *Cronobacter* specific antigen

Finding *Cronobacter* specific antigen: We used pAb of *Cronobacter* cell to find its specific antigen through western blotting. Six bacteria (*C. sakazakii* ATCC 29544, *C. muytjensii* ATCC 51329, *C. malonatics* CMCC 45402, *C. sakazakii* YC633B, *C. muytjensii* SR1074B and *C. malonatics* JA264B) and *Salmonella typhimurium* ATCC 13311 as control were applied in this study. Stock culture were inoculated into LB medium and cultured overnight at 37°C with shaking. After centrifugation (4000 g, 5 min), bacteria were collected and washed twice by PBS buffer. After boiling with loading buffer (10 min), the bacteria were sampling through western blotting. Using pAb against *Cronobacter* cell, goat-anti-rabbit IgG-HRP and DAB for coloration. Four bands that were the same in six lanes were found to be specific protein of *Cronobacter* after coloration and further used for specific antigen.

Construction of the expression vector: The genomic DNA of *C. muytjensii* ATCC 51329 was extracted by using Bacteria Genomic DNA Extraction Kit (Takara, Japan). The gene specific primers for amplification of this gene were designed according to the gene sequence obtained from GenBank. The following primers were used: forward, 5'-TTCGAATTCCCTGGCGCTTACTGGTGG-3'; reverse, 5'-GAGCTCGAGTTAGTACGTCGTCGGGGCC-3'. The PCR product was purified using SanPrep Column DNA Gel Extraction Kit (Sangon, China) and digested with EcoR I/Xho I ligated into the pGEX-4T-1 vector. Then the recombinant plasmids were transformed into the electrochemistry competent *E. coli* JM109 cell for protein expression under the induction with isopropyl-β-D-thiogalactopyranoside (IPTG, 0.8 mM, 30°C, overnight). The expressed protein was assayed by SDS-PAGE and western blotting.

Purification of the fusion protein: CP783 fusion protein was expressed in electrochemistry competent *E. coli* JM109 cell under the induction with IPTG. According to the result of the western blotting, we chose the purified protein CP783 as *Cronobacter* specific antigen. The CP783 protein was purified by using Profinia TM GST Kit (Bio-Rad, American).

Animal immunization: Six to eight week old, male mice were given a series of subcutaneous (s.c.) injections as follows: mice were injected every 14 days and 5 times in total, 150 μg purified CP738 protein for each. The mice were bled 3 days later, and the serum obtain was kept frozen at -20°C or -80°C in small aliquots of 1-5 ml. The titer of serum antibody was determined by Elisa assay, the specificity of serum antibody was assayed by dot blot analysis. Serum antibody was purified by bacterial adsorption and saturated ammonium sulfate [$(NH_4)_2SO_4$].

Preparation of the fluorescent microsphere-pAb conjugate

The density of carboxyl around the fluorescent microsphere used in this study was 625 mM/g. We used pAb against CP783 to couple fluorescent microsphere. Briefly, Coupling was initiated by the addition of 25 μg of pAb against recombinant CP783 protein and 5 mg activated fluorescent microsphere, brought to a final volume of 9 ml with 0.01 M PBS and incubated with EDC for 2 h at room temperature with rotation. Coupled microspheres were blocked to reduce nonspecific binding by the final concentration 1% of BSA solution for 30 min. Coupled microspheres were harvested by centrifugation (8000 rpm, 5 min) then washed once with 10 ml of 0.01 M PBS and re-suspended by 2 ml 0.01 M PBS, stored in 4°C until use.

Preparation of the optimal concentration of the coating antibody

The pAb against *Cronobacter* cell mixture were diluted to 3.0, 2.5, 2.0, 1.5, 1.0, and 0.5 mg/ml with 0.02 M sodium PBS (pH 8.5). The diluted pAb and 1 mg/ml goat anti-rabbit IgG were transferred onto the NC membrane with a volume of 1 μl/cm to form the test (T) and the control (C) lines, respectively. The distance between the T and C lines was 8 mm. The test strips were dried at 37°C for 8 h. In this study, 0.5% OVA were used as the blocking buffer.

Preparation of the immunochromatographic strip

The structure of the immunochromatographic test strip was described in **Figure 1**. 700 μL of 2.0 mg/mL pAb against the *Cronobacter* whole proteins was dispensed onto the lower part of a nitrocellulose membrane strip with a Bio-strip Dispenser HGS102, as the test line (T), while 500 μL of 1.0 mg/mL goat anti-rabbit IgG was dispensed 8 mm above the test line, as the control line (C). The fluorescent microsphere labeled pAb against CP783 protein (10 uL/cm) was added to the conjugate pad at the speed of 8 μL/cm and then dried at 30°C for 2 h in vacuum, the nitrocellulose membrane was blocking in 0.5% OVA for 10 min, then continued drying.

Sample pad made of glass fiber was treated with PBS (pH 7.4) containing 1% BSA, 1% trehalose, 0.02% sodium azide and 0.1% Tween 20 and dried at 37°C. Absorption pad made of filter paper was applied immediately without pretreatment. The sample pad, conjugate pad, immobilized NC membrane, and absorbent pad were assembled, as described in **Figure 1A**. These strips were cut into 5 mm width by BIODOT and stored in a desiccator at 4°C for future use. After the samples (100 µl) were added drop by drop to the sample pad and allowed to pass through the NC membrane. After 10 min, the appearance of two light lines in the test and control line was positive result. The negative result was the appearance of only one light line in the control line. The appearance of only a single light line in the test line or the absence of a line in test strip was confirmed an invalid test (**Figure 1B**).

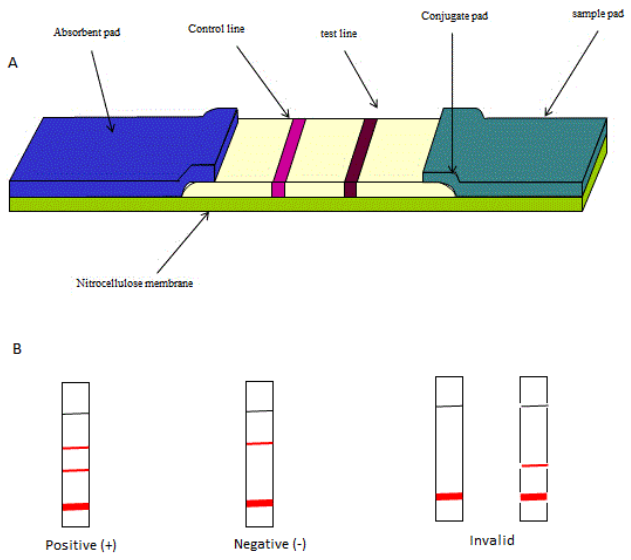

Figure 1: (A) The schematic diagram of the immunochromatographic strips and (B) The illustration of immunochromatographic test results.

Sensitivity and stability of the immunochromatographic strip

Different concentrations of the pure culture and milk samples containing *Cronobacter* (10^8 CFU/mL, 10^7 CFU/mL, 10^6 CFU/mL, 10^5 CFU/mL, 10^4 CFU/mL and 10^3 CFU/mL) were prepared with 0.01 M sterile PBS buffer (pH 7.2) to evaluate the sensitivity of the immunochromatographic strip. For the sensitivity assay, 100 µl of each solution of a particular dilution was used for the immunochromatographic strip test and 0.0l M PBS buffer (pH 7.2) was used for the blank control. All immunochromatographic strips were stored at 4°C for 16 weeks to evaluate the stability of the immunochromatographic strips during storage.

Detection of simulated milk powder

The pure cultured bacteria of *Cronobacter* were diluted to different concentration (10^8 CFU/mL, 10^7 CFU/mL, 10^6 CFU/mL, 10^5 CFU/mL, 10^4 CFU/mL and 10^3 CFU/mL) using aseptic milk. Then, high concentration *Salmonella typhimurium* ATCC 13311 (10^8 CFU/mL) was added to the samples. 100 µl of each solution of a particular dilution was used for the immunochromatographic strip test, and aseptic milk was used as the blank control.

Detection of large sample

A total of 100 samples containing 80 samples have *Cronobacter* diluted to 10^7 CFU/ml with milk and other milk samples had no *Cronobacter* for negative. All samples were also detected by the ELISA kit to evaluate the accuracy of the immunochromatographic strip test.

Results and Discussion

Specific test of pAb against *Cronobacter*

We used 55 bacteria (**Table 1**) to detect the specific of pAb, cross reaction was seen between pAb and 32 *Cronobacter*.

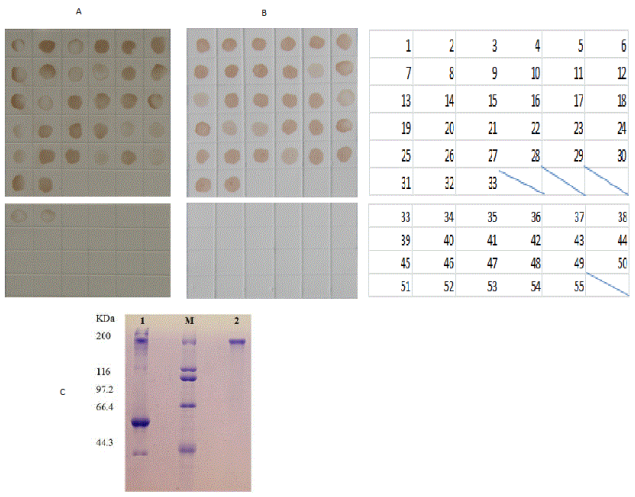

Figure 2: Detection for specific of pAb against *Cronobacter* whole protein by dot blot and SDS-PAGE. (A) Before purification, there are cross-reaction between the pAb with No.33 and No.34 bacterium, (B) After purification, the pAb can only combine with *Cronobacter*. No cross-reaction with others and (C) Line 1, pAb before purification; Line M, protein marker; Line 2, pAb after purification. According to these results, the pAb against *Cronobacter* that is high specificity was confirmed.

But pAb against CP783 protein also had reaction with two other pathogens (*Vibrio parahaemolyticus* PVPA 0146 and *Salmonella typhimurium* ATCC 13311). The results were shown in **Figure 2A**. After purification by Bacterial adsorption method and saturated ammonium sulfate [$(NH_4)_2SO_4$], the SDS-PAGE and dot blot result showed that the purified pAb can also combine with 32 *Cronobater*, but not with other bacteria besides *Cronobacter* (**Figure 2B**).

Specific test of pAb against recombination CP783 protein

Through western blotting assay, we confirmed the four genes of those proteins, ATPsyn (GI 156936114), Maltoporin (GI 156932308), OmpA (GI 156934557), CP783 (GI 156934989). According to NCBI database, the DNA sequence of OmpA is highly similar to other bacterium, like *Salmonella typhimurium*. Through MS/MS assay and blast with NCBI database, the protein sequence of Maltoporin and OmpA were also similar with other bacteria which were not *Cronobacter*. So, we chose CP783 for cloning, expression (**Figure 3**) and purified (**Figure 4**).

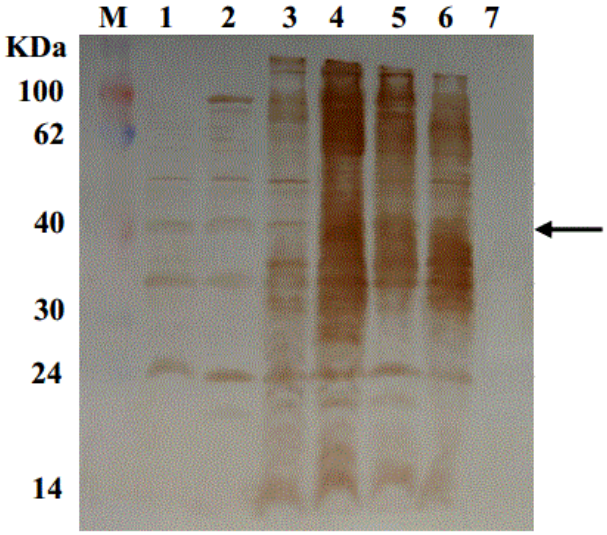

Figure 3: The results of CP783 protein expression and purification: (A) M, marker; 1, *C. sakazakii* YC633B; 2, *C. malonatics* JA264B; 3, *C. muytjensii* SR1074B; 4, *C. sakazakii* ATCC 29544; 5, *C. muytjensii* ATCC 51329; 6, *C. malonatics* CMCC 45402; 7, *Salmonella typhimurium* ATCC 13311. The CP783 protein was common exist in *Cronobacter* and chose to specific antigen of *Cronobacter*.

After cloning and expression, purified CP783 protein injected mouse, then the serum was harvested and estimated the specific of serum by dot blot. The result showed that the pAb could combine 32 *Cronobacter* in our laboratory, but the positive reaction was seen between pAb and 4 other bacteria, *Salmonella typhimurium* ATCC 13311, Enteropathogenic *E.coli* CMCC 44496, *Esherichia coli* O157:H7 PELI 0480, *Shigella flexneri* ATCC 29903 (**Figure 5A**). After purification using the 4 bacteria by bacterial adsorption and saturated ammonium sulfate [$(NH_4)_2SO_4$], the SDS-PAGE and dot blot result showed that the purified pAb can also combine 32 Cronobater, but not other bacteria (**Figure 5B**).

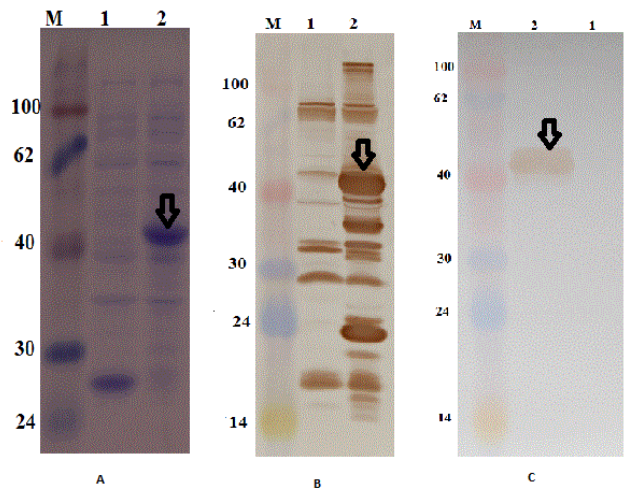

Figure 4: (A) SDS-PAGE for detection of CP783 protein expression; (B) Western Blot by using unpurified pAb of *Cronobacter* cell as primary antibody; (C) Western Blot by instead of purified pAb of *Cronobacter* cell. Line M, maker; Line 1, negative control (*E. coli* JM109 with empty vector); Line 2, CP783 protein expression (*E.coli* JM109 with vector carrying CP783 gene), those results confirmed the CP783 protein was expressed and purified successfully.

Optimal concentration of the capture antibody, coating antibody, and blocking BSA

The pAb using CP738 protein as immunogen was produced by the method described above. The most advantageous concentration of the capture antibody was 20 ug/ml. for the coating antibody, the color of the test line depended gradually with the increase in pAb concentration. But, the color of the test line was maintained when the pAb concentration reached 2 mg/ml. In this study, 1% BSA solution is best for blocking buffer, thus, 1% BSA solution was determined as the most advantageous concentration of the blocking buffer.

Sensitivity of the immunochromatographic strip

To confirm the sensitivity of the immunochromatographic strip, different concentrations of the pure culture and milk samples containing *Cronobacter* (10^8 CFU/ml, 10^7 CFU/ml, 10^6 CFU/ml, 10^5 CFU/ml, 10^4 CFU/ml, 10^3 CFU/ml) were prepared for test. The results are shown in **Figure 6**. The test was repeated 3 times and the results showed that the bacteria minimum concentration can be detected to reach 10^5 CFU/ml in pure culture and 10^6 CFU/ml in milk. No cross-reaction was observed when other samples were tested.

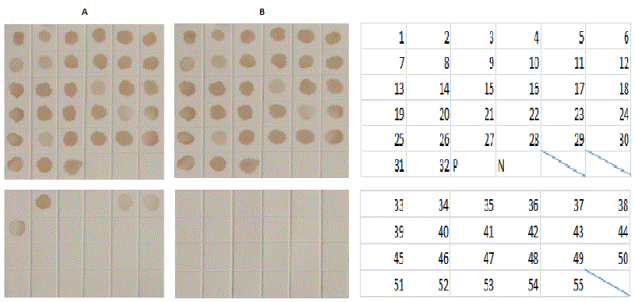

Figure 5: Detection for specific of pAb against CP783 protein by dot blot: (A) Before purification, the pAb can combine with other bacteria (No.34, 37, 38, 39), (B) After purification, the pAb can reaction with *Cronobacter* specially. Those results confirmed that the pAb against CP783 protein was high specific and had no cross-reaction with other bacteria which was not *Cronobacter*.

Stability of the immunochromatographic strip

The same batch of strips was stored at 4°C for 16 weeks to estimate their stability by evaluating the sensitivity and specificity. The results demonstrated the strips were stable at 4°C for 16 weeks at least.

Detection of simulated milk powder and large sample

For detection of simulated milk powder, the result was shown in **Figure 7**. There were positive reactions for test group, and there were negative reactions for the blank groups. These results confirmed our immunochromatographic strips can detect *Cronobacter* specifically, and large number of other bacteria cannot affect the ability for detecting *Cronobacter*.

For large sample detection, 79 samples were detected for positive reaction in 80 samples containing *Cronobacter*. Moreover, all negative reactions were shown for 20 blank samples. In the end, a rapid detection method of *Cronobacter* was well established by immunochromatographic strip which can detect 32 *cronobacter* in our laboratory. It had also high specificity and sensitivity to detect *Cronobacter* in milk, thus can better ensure the safety of milk, especially for the powdered infant formula.

Conclusion

We developed a fluorescent microsphere-based immunochromatographic strip with high specificity and sensitivity. It can detect at very low concentration of *Cronobacter* in milk. The minimum detectable concentration of *Cronobacter* was found to be at 10^5 CFU/ml in pure culture and 10^6 CFU/ml in milk.

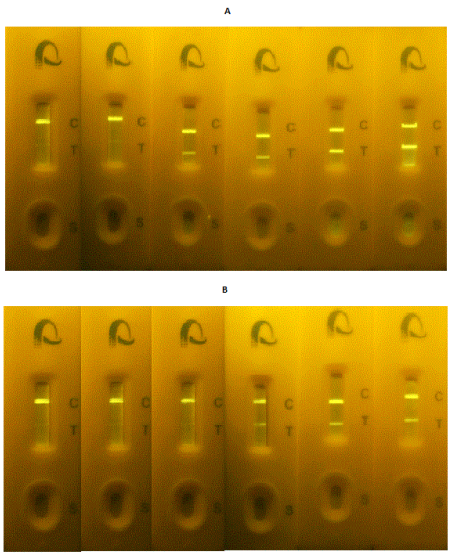

Figure 6: Sensitivity of the immunochromatographic strip. Different diluted concentrations of *Cronobacter* solutions respectively in PBS and milk. (1, 10^3 CFU/mL; 2, 10^4 CFU/mL; 3, 10^5 CFU/mL; 4, 10^6 CFU/mL; 5, 10^7 CFU/mL and 6, 10^8 CFU/mL), (A) Sensitivity of the immunochromatographic strip in pure culture of *Cronobacter* can reach 10^5 CFU/mL in this study, (B) Sensitivity of the immunochromatographic strip in milk samples of *Cronobacter* can reach 10^6 CFU/mL.

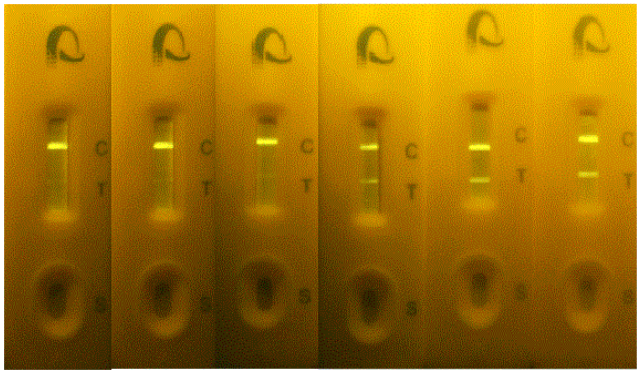

Figure 7: Detection of simulated milk powder. Different diluted concentrations of *Cronobacter* solutions in milk which mixed with 10^8 CFU/mL *Salmonella typhimurium* ATCC 13311; 1, 10^3 CFU/mL; 2, 10^4 CFU/mL; 3, 10^5 CFU/mL; 4, 10^6 CFU/mL; 5, 10^7 CFU/mL and 6, 10^8 CFU/mL. It was clarified that other bacterium cannot affect the reaction about the immunochromatographic strip.

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