Highly sensitive detection of Cronobacter sakazakii based on immunochromatography coupled with surface-enhanced Raman scattering

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ABSTRACT

The presence of Cronobacter sakazakii must be controlled in infant powder plants, because it may cause infectious disease in infants, with high mortality. Testing for C. sakazakii in powdered infant formula should be performed before delivery, and it requires rapid and specific detection methods. In this study, we established a surface-enhanced Raman scattering (SERS) immunochromatographic test strip for the quantitative determination of C. sakazakii in powdered infant formula. Monoclonal antibodies for C. sakazakii were labeled with p-aminothiophenol-bound colloidal gold nanoparticles. Color change in the test line indicated the presence of C. sakazakii. A highly sensitive and quantitative test method was developed based on the Raman signal produced by the p-aminothiophenol bonding on gold nanoparticles. The SERS immunochromatographic test strip assay required a short analysis time (12 min) and exhibited a linearity range from 10^2 to 10^7 cfu/mL. The limit of detection was 201 cfu/mL without preculture. The SERS immunochromatographic test strip assay is a promising tool for the simple and rapid quantitative analysis of C. sakazakii and other pathogenic bacteria.

Key words: Cronobacter sakazakii, surface-enhanced Raman scattering, immunochromatographic test strip, colloidal gold nanoparticles

INTRODUCTION

Cronobacter sakazakii, formerly Enterobacter sakazakii (Monroe and Tift, 1979; van Acker et al., 2001; Iversen et al., 2007), is a pathogen that causes infectious diseases such as bacteremia, neonatal meningitis, and necrotizing enterocolitis, associated with high mortality rates in infants. Powdered infant formula is frequently contaminated with C. sakazakii (Drudy et al., 2006; Baumgartner et al., 2009). The European Union requires that Cronobacter spp. be absent from foodstuffs (European Commission, 2005) in powdered infant formula. Therefore, a rapid and sensitive detection method is urgently needed by industries and regulatory agencies for the detection and quantification of C. sakazakii (ISO, 2017).

Cronobacter sakazakii has been detected using traditional selective culture methods, biochemical reactions, and PCR technology (Minami et al., 2012; ISO, 2017; de Benito et al., 2019; Akineden et al., 2020), but these conventional methods are laborious and time-consuming. Indeed, PCR-based methods require total DNA extraction and target DNA amplification, which may result in false positives (Friedemann, 2007), and they require several hours for detection (Srikumar et al., 2019; Yuan et al., 2020). Real-time fluorescence PCR has been developed to quantify C. sakazakii, but it requires technical proficiency and comes with high costs (Tutar et al., 2018).

The immunochromatographic test strip, based on immunological recognition, has attracted increasing attention because of its high specificity and ease of use in on-site testing (Aragay et al., 2012; Hu et al., 2014; Koczula and Gallotta, 2016). However, the sensitivity and accuracy of the test strip need to be confirmed for pathogen detection (Scharinger et al., 2017; Pan et al., 2018). In test strip assays, gold nanoparticles (AuNP) are used to label the antibody, resulting in color changes upon interaction of the antibody with antigens (Bu et al., 2019; Wu et al., 2020). However, before color changes are observed, the antigen–antibody combination has already occurred (Sajid et al., 2015); more sensitive signals of antigen–antibody combination are needed.

In the 1970s, individual silver colloidal nanoparticles were used to obtain a 10^14 to 10^15-fold amplification of the Raman signal from single rhodamine 6G molecules.
comparing to conventional detection methods. The Raman signals showed higher stability and strength on colloidal nanoparticles compared to single molecules (Nie and Emory, 1997). Light resonance stimulates electromagnetic molecules and enhances the signals by 10²- to 10³-fold. Due to resonance, the surface-enhanced Raman scattering (SERS) effects of single molecules may yield a 10⁴- to 10⁵-fold signal amplification (Hwang et al., 2016).

Nanoscale levels of colloidal elements such as Ag and Au can form plasmonic nanoparticles and magnify the signal by resonance absorption or incident light scattering (Anker et al., 2008; Lombardi and Birke, 2009; Zong et al., 2018). Nanoscale colloidal particles amplify Raman spectroscopy signals by combining with incident light energy (Aizpurua et al., 2003; Zong et al., 2018). Previously, SERS sensitivity has been applied in immunochromatographic assays (Deng et al., 2019; Li et al., 2019). However, SERS-based strips have rarely been used to detect pathogens. Because of the large size of bacteria compared to other antigens, the preparation of colloidal particles with Raman molecular and strip detecting conditions should be carefully optimized.

In this study, a new SERS-based immunochromatographic test strip was developed for the simple and rapid quantification of C. sakazakii. Our method relied on the use of Raman reporter gold nanoparticles instead of the traditional gold nanoparticles used in typical test strip analyses. The Raman signal of SERS nanotubes was monitored to achieve high sensitivity and quantitative assessment of C. sakazakii. This new SERS immunochromatographic test strip assay is a promising tool for the rapid and simple quantitative detection of C. sakazakii.

MATERIALS AND METHODS

Chemicals and Instruments

Chloroauric acid (HAuCl₄), sodium citrate (Na₃citrate), p-aminothiophenol (PATP), goat anti-rat IgG, and BSA were purchased from Sigma-Aldrich (St. Louis, MO). Anti-C. sakazakii monoclonal antibodies were prepared by the laboratory. Powdered infant formula was purchased from YeLi Dairy (Heilongjiang, China). Polyvinylpyrrolidone was purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Absorbent pads, sample pads, and nitrocellulose membranes were purchased from Sartorius Trading Co. Ltd. (Shanghai, China). All other chemicals were analytical-grade reagents. Ultraviolet-visible absorption spectra were assayed by using a Lambda 35 UV/VIS spectrometer (PerkinElmer, Waltham, MA). Transmission electron microscopy images were captured with an HT7800 transmission electron microscope (Hitachi, Tokyo, Japan). Raman spectra were obtained using a Raman microspectrometer system (inVia Raman microscope; Renishaw, Wotton-under-Edge, UK).

Bacterial Strains

We used 3 strains of C. sakazakii ATCC 29544, 12868, and 29004, as well as Cronobacter muytjensii ATCC 51329, Cronobacter universalis NCTC 9529, Staphylococcus aureus ATCC 25923, Salmonella typhimurium ATCC 14028, Shigella flexneri ATCC 29931, Listeria monocytogenes ATCC 19114, and Escherichia coli ATCC 25922. All strains were cultured in Luria-Bertani broth (Aladdin China Ltd., Shanghai, China) for 24 h at 37°C.

Preparation of Monoclonal Antibodies

Cronobacter sakazakii ATCC 29544 was activated and subcultured in at 37°C for 24 h. The C. sakazakii cells were centrifuged at 5,000 × g for 10 min. The pellets were ultrasonically disrupted 70 times for 5 s at 350 W with 9-s intervals at 26°C (VCX 605; Sonics, Newton, CT). The lysates were extracted with equal amounts of 90% phenol, kept at 68°C for 30 min, and dialyzed using dialysis tubing of 6,000 to 8,000 molecular weight (Biomol GmbH, Hamburg, Germany) at room temperature against distilled water for 48 h. The dialysate was concentrated with polyethylene glycol 20000 and purified to obtain crude LPS, which was used to immunize 8-wk-old BALB/c female mice with Freund’s complete adjuvant emulsion, followed by 5 subsequent immunizations at 2-wk intervals (Scharinger et al., 2016). After the mice were killed, spleen lymphocytes were collected and fused with myeloma cells at a 5:1 ratio in polyethylene glycol 1500. Hybridoma cells with high antibody activity were selected, cloned in RPMI-1640 medium with 10% calf serum, and stored frozen in liquid nitrogen. Another group of 8-wk-old female BALB/c mice were injected intraperitoneally with 0.5 mL of paraffin. After 7 d, the hybridoma cells were resuspended in RPMI-1640 medium and intraperitoneally injected (Wu et al., 2015; Scharinger et al., 2016). The ascitic fluid was then collected and the mAb were purified using the caprylic acid-ammonium sulfate method (Shi et al., 2018). A mouse mAb Ig subclass detection kit (Thermo Scientific, West Palm Beach, FL) was used to determine the antibody subtype (Jaradat et al., 2011).

Preparation of SERS Immunoprobe

Preparation of the SERS immunoprobe was performed as previously described, with minor modifications...
Briefly, the immunoprobe (mAb-Au-PATP) was assembled by sequential addition of the Raman reporter (PATP) and the antibody to colloidal gold particles. Colloidal gold particles were prepared using the citrate reduction method (Liang et al., 2014b). Ultrapure water was heated to boiling, and HAuCl4 was added to obtain 300 mL of 0.01% aqueous solution. Next, 4.5 mL of 1% trisodium citrate solution was added to the boiling solution for 15 min to obtain colloidal gold particles, and the colloidal solution was cooled to room temperature. Then, 0.1 mol/L K2CO3 was added to the mixture to adjust the pH to 9.0 before addition of PATP. Then, 10 mL of the colloidal gold solution was mixed with 10 μL of 1 mmol/L PATP solution and stirred at 25°C for 15 min. The mixture was kept at 4°C for 1 h, and the mAb against C. sakazakii were added to 1 mL of PATP-labeled colloidal gold particle solution, followed by stirring at room temperature for 15 min and cooling at 4°C for 1 h to obtain the mAb-Au-PATP preparation. To block nonspecific binding sites, 100 μL of 5% BSA was added to the solution and kept overnight at 4°C. Finally, mAb-Au-PATP was obtained by centrifuging at 5,000 × g for 10 min, and resuspended with PBS (0.5 M, pH 7.4) to the original volume. The final mAb-Au-PATP solutions were stored at 4°C.

Figure 1. Schematics of the surface-enhanced Raman scattering (SERS) immunochromatographic test strip assay for the detection of Cronobacter sakazakii. (A) Preparation and structure of the mAb-Au-PATP probe. (B) Assembly of the strip and SERS immunochromatographic test strip procedure for C. sakazakii detection. AuNP = gold nanoparticle; C = control; NC = nitrocellulose; PATP = p-aminothiophenol; T = test.
Assembly of the Lateral Flow Strip

The immunochromatographic strip was assembled using a nitrocellulose membrane, sample pad, and absorbent pad on a polyvinyl chloride plate (Figure 1B). Then, 10 μL of 1 mg/mL mAb was layered on the nitrocellulose membrane to form the test line, and 10 μL of goat anti-rat IgG was layered to form the control line. The membranes were dried at room temperature for 1 h. A glass-fiber membrane, serving as a sample pad, was soaked in PBS for 24 h (0.5 M, pH 7.4, containing 20% polyvinylpyrrolidone, 20% Tween-20). A nitrocellulose film was adhered to the polyvinyl chloride board. The sample and absorbent pad were attached to each end of the nitrocotton film, with an overlap of 1.5 mm. Then, the attached films were cut into strips 4 mm wide. The strips were sealed in glass vials and stored at 4°C. The immunochromatographic strips were directly dipped into wells containing mAb-Au-PATP and sample solutions.

SERS Immunochromatographic Test Strip Procedure

Detection using the SERS immunochromatographic test strips was carried out by immersing the sample pad in the mAb-Au-PATP solution containing 100 μL of the sample. The sample solution, together with the SERS immunoprobes, flowed onto the absorption pad. The reaction was considered complete when the control line turned red. The Raman signal reflected the extent of Ag-mAb-Au-PATP/antibody complex formation on the test line. The resonance was excited using a 10 mW 785 nm laser with an integration time of 10 s. The Raman spectrum between 800 and 1,900 cm⁻¹ was analyzed. The intensity at 1,081 cm⁻¹ of 10 different points on the test line was selected for quantitative calculation of signal intensity.

Quantitative Calculation of Signal Intensity

Commercial powdered infant formula free of C. sakazakii was used to generate artificially contaminated samples. First, 10 g of powdered infant formula was reconstituted with 90 mL of buffered peptone water preheated to 44°C, and C. sakazakii bacteria were added at final concentrations between 10⁸ and 10² cfu/mL. Then, the plate counting method was used to establish the initial concentration of the C. sakazakii standard suspension. The samples were centrifuged for 10 min at 4,500 × g and 4°C, and the cell pellets were suspended in normal saline. Then, 100 μL of the suspended solution was loaded onto the SERS immunochromatographic test strip for detection.
Optimization of SERS Immunochromatographic Test Strip

To improve the performance of the SERS immunochromatographic test strip, several key parameters were optimized, including mAb concentration and assay time, using the bacterial concentration of 10^6 cfu/mL. Because the concentration of anti-C. sakazakii mAb on the test line had a great influence on detection sensitivity, this parameter was optimized first. As shown in Figure 3A, at mAb concentrations exceeding 1 mg/mL, strong Raman signals were observed from positive reactions, and low background signals were detected in negative reactions (PBS). Therefore, 1 mg/mL of anti-C. sakazakii mAb was identified as the optimal concentration.

Assay time was also crucial for optimal performance of the SERS immunochromatographic test strip. As shown in Figure 3B, the intensity at 1081 cm\(^{-1}\) increased steadily until 12 min, and then reached a plateau. Therefore, an assay time of 12 min was adopted. The appropriate amount of anti-C. sakazakii mAb for Au-PATP labeling was also investigated, and the highest Raman signal was obtained with a concentration of 10 μg/mL (Figure 3C).

Quantitative Detection of C. sakazakii

To define the detection limit of the SERS immunochromatographic test strip for C. sakazakii analysis, the assay was used to test bacterial concentrations of 0 to 10^7 cfu/mL (Figure 4A). The color of the test line became stronger with increases in C. sakazakii concentration. The concentration limit could be visually assessed and was determined to be 10^5 cfu/mL.

Bacteria combined with mAb-AuNP-PATP immunoprobes and antigen–antibody complexes were captured by the coated antibodies on the test line. Raman signal intensity increased with the accumulation of PATP in the bacteria–mAb-AuNP-PATP complexes. Therefore, Raman signal intensity increased with C. sakazakii concentration (Figure 4B). A bacterial concentration of 10^2 cfu/mL yielded a Raman signal intensity of 1,126.87 ± 24.90 a.u., significantly higher than the blank (957.51 ± 57.28 a.u.; \(P = 0.04\)). Quantitative analysis revealed a nonlinear calibration curve (Figure 4C). The limit of detection was estimated to be 201 cfu/mL, calculated by 3 times the standard deviation in the presence of 0 cfu/mL. The strip showed positive results when the sample with C. sakazakii was enriched at 37°C for 18 h. Under these conditions, the bacterial titer reached approximately 201 cfu/mL in buffered peptone water broth according to the ISO 22964:2017 method (ISO, 2017). With the precultivation procedure, the SERS immunochromatographic test strip assay may be a suitable method for detecting C. sakazakii infection in industrial plants.

The antibody-conjugated SERS nanoparticles significantly improved the limit of detection of the strip, increasing its sensitivity by approximately 2 orders of magnitude.
magnitude compared to that achieved by visual assessment of color changes.

**Reproducibility and Selectivity of the SERS Immunochromatographic Test Strip Method**

To assess the reproducibility of the SERS-based method, 10 independent measurements of Raman signal intensity at 1,081 cm\(^{-1}\) were performed at each *C. sakazakii* concentration from \(10^7\) to \(10^3\) cfu/mL (Figure 5A). Signal intensity was monitored in the middle of the test lines. Relative standard deviations were 3.4, 2.8, 5.2, 5.5, and 5.3% for the different bacterial concentrations, respectively, suggesting high precision. Reproducibility was similar to the technology applied to detect other hazardous substances (Zong et al., 2018). The relative standard deviation was lower than the visible light signal of the strip (Wang et al., 2012; Akineden et al., 2020).

The strain used to obtain the antibody was *C. sakazakii* ATCC29544. Because other *Enterobacter* spp. may occur concurrently with *C. sakazakii*, common foodborne pathogens were used to examine the specificity of the SERS strip. To guarantee the specificity of the strip developed, mAb were used instead of polyclonal antibodies. For high sensitivity, lipopolysaccharide was used as an adjuvant to obtain mAb. To verify the specificity of the mAb obtained, *C. sakazakii* ATCC128868, *C. sakazakii* ATCC 29004, *C. muytjensii* ATCC 51329, *C. universalis* NCTC 9529, *Staphylococcus aureus* ATCC 25923, *Salmonella typhimurium* ATCC 14028, *Shigella flexneri* ATCC 29931, *L. monocytogenes* ATCC 19114, and *E. coli* ATCC 25922 were used to verify the specificity of the mAb obtained. A competitive ELISA method was used to detect the specificity of the mAb. As listed in Table 1, the 3 strains of *C. sakazakii* showed positive results, and the 7 strains of other bacteria showed negative results. No cross-reactivity with other pathogens was found (Table 1). These results indicated that the SERS strip developed in our study was specific to *C. sakazakii*. The SERS strip had high specificity for *C. sakazakii* and no cross-reaction to *Staphylococcus aureus*, *Salmonella typhimurium*, *Shigella flexneri*, *L. monocytogenes*, or *E. coli*. The SERS strip had the same specificity for the mAb used in the strip (Figure 5B). Strains of *C. sakazakii* gave out Raman signals, but the other bacterial strains did not. These results were consistent with those of Pan et al. (2018), except that the strip tested in the present study showed no cross-reaction with *Staphylococcus aureus*, different from the report of Pan et al. (2018). The reason for this may have been that the lipopolysaccharide adjuvant
evaluated the specificity of the antibody generated (Scharinger et al., 2017).

**Application of SERS Immunochromatographic Test Strip for Detection of C. sakazakii**

To verify the accuracy of the method, the SERS immunochromatographic test strip was applied to real samples. The amount of *C. sakazakii* in milk powder (2 × 10^5 to 1.6 × 10^6 spiked-in bacteria) was measured using the SERS immunochromatographic test strip; the curves obtained are summarized in Figure 6. Concentrations of *C. sakazakii* were also determined using the plate counting method, which is currently the gold standard (Table 2). Test accuracy was calculated based on the ratio between the values obtained using the SERS immunochromatographic test strip and those obtained by plate counting. The results showed accuracy in the range of 99 to 105%, demonstrating that the SERS immunochromatographic test strip assay can be used for the accurate measurement of *C. sakazakii* titers in milk powder. The limit of detection was 201 cfu/mL, and the concentration limit for visual detection was 10^5 cfu/mL. As well, using an enrichment procedure at 37°C for 18 h, this method could detect 1 cfu/100 g, which was more sensitive than the ELISA method (Song et al., 2018).

**CONCLUSIONS**

The SERS immunochromatographic test strip assay proved to be highly specific for the detection of *C. sakazakii*. 

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*Figure 4. Quantitative performance of the surface-enhanced Raman scattering (SERS) immunochromatographic test strip assay. (A) Color intensity of the test (T) line as a function of *Cronobacter sakazakii* concentration. (B) SERS spectra of the T line. (C) Calibration curves of SERS signal as a function of *C. sakazakii* concentration; values represent the mean of 3 replicates (n = 3). C = control. Error bars indicate SD.*
zakii; it was also highly sensitive, because of the Raman signal. In this system, the presence of bacteria could be visually assessed in 12 min based on color change in the test line. The intensity of the Raman signal produced by the mAb-Au-PATP immunoprobes on the test line was highly correlated with bacterial concentration in the samples, providing accurate quantification of the C. sakazakii titers. The range of quantitative detection was $10^2$ to $10^7$ cfu/mL, with a limit of detection of 201 cfu/mL and an accuracy of 99 to 104%, compared to plate counting without preculture. Based on the ISO 22964:2017 enrichment procedure, the method met the detection requirements of the European Commission.

Because of its short assay time and ease of implementation, the SERS immunochromatographic test strip assay described in this article proved to be an effective tool for simple and rapid quantification of specific pathogens in industrial food.

**ACKNOWLEDGMENTS**

This work was financially supported by the Heilongjiang Province University Basic Research Operating Costs Heilongjiang University Special Project (no. KJCX201817). Siyuan Gao performed the experiments and wrote the manuscript draft; Jinhui Wu helped with the monoclonal antibody experiments; Hong Wang and Shengying Hu helped with data analysis; Li Meng designed the experiments, provided guidance, and finalized the manuscript. The authors have not stated any conflicts of interest.

**Table 1.** Specificity of mAb testing (by competitive ELISA) and the surface-enhanced Raman scattering (SERS) immunochromatographic test strip assay

<table>
<thead>
<tr>
<th>No.</th>
<th>Strain</th>
<th>mAb</th>
<th>SERS</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Cronobacter sakazakii ATCC 29544</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Cronobacter sakazakii ATCC 12868</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Cronobacter sakazakii ATCC 29004</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Cronobacter muytjensii ATCC 51329</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>5</td>
<td>Cronobacter universalis NCTC 9529</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>6</td>
<td>Staphylococcus aureus ATCC 25923</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>7</td>
<td>Salmonella typhimurium ATCC 14028</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>8</td>
<td>Shigella flexnerii ATCC 29931</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>9</td>
<td>Listeria monocytogenes ATCC 19114</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>10</td>
<td>Escherichia coli ATCC 25922</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

+ = positive; − = negative.

**Table 2.** Detection of Cronobacter sakazakii in infant milk powder using the plate counting method and the surface-enhanced Raman scattering (SERS) immunochromatographic test strip assay (mean ± SD; n = 5)

<table>
<thead>
<tr>
<th>Plate counting method (cfu/mL)</th>
<th>SERS (cfu/mL)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>255 ± 23</td>
<td>280 ± 24</td>
<td>105 ± 9</td>
</tr>
<tr>
<td>456 ± 38</td>
<td>473 ± 108</td>
<td>104 ± 5</td>
</tr>
<tr>
<td>18,207 ± 907</td>
<td>18,133 ± 808</td>
<td>99 ± 3</td>
</tr>
<tr>
<td>1,650,000 ± 75,498</td>
<td>1,645,178 ± 30,970</td>
<td>100 ± 3</td>
</tr>
</tbody>
</table>
REFERENCES


Figure 6. Results of sensitivity test for spiked sample. In spiked samples 1, 2, 3, and 4, the amounts of Cronobacter sakazakii added were 255 ± 23, 456 ± 38, 18,267 ± 907, and 1,650,000 ± 75,498 cfu/mL, respectively, using the plate counting method.


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