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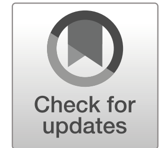
RESEARCH PAPER



Rapid detection of trace *Salmonella* in milk and chicken by immunomagnetic separation in combination with a chemiluminescence microparticle immunoassay

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Abstract

Rapid detection of trace *Salmonella* is urgently needed to ensure food safety. We present an innovative pretreatment strategy, based on a two-step enrichment culture and immunomagnetic separation, combined with a chemiluminescence microparticle immunoassay to detect at least one proliferative *Salmonella* cell in 25 mL (25 g) food. The capture performance of immunomagnetic beads (IMBs) of sizes for *Salmonella* was investigated, and the IMBs of size 2.8 μm showed a high capture efficiency of 60.7% in 25 mL milk and 74.5% in 25 mL chicken culture filtrate, which ensured the successful capture of trace *Salmonella* after 2.5 h in situ enrichment even from only one *Salmonella* cell. The separated *Salmonella* cells, reaching an amount of 10^3 colony-forming units (CFU) by a secondary enrichment for 3 h, were detected by a horseradish peroxidase chemiluminescence reaction with 4-(1-imidazolyl)phenol as an enhancer, which evidenced a linear response for *Salmonella* concentrations ranging from 2.3×10^2 to 7.8×10^4 CFU/mL. The entire detection process was completed within 8 h, with a very low detection limit of 1 CFU/25 mL (25 g), which was verified by colony counting, and a small degree of interference of 0.17–1.06%. Trace *Salmonella* from five different serovars in milk and chicken was successfully detected without false negative or false positive results. Furthermore, this study provides a basis to develop a fully automated instrument based on IMBs that includes all steps from sample preparation to chemiluminescence microparticle immunoassay for high-throughput screening of foodborne pathogens.

Keywords Pretreatment · Pathogen · Chemiluminescence immunoassay · Enrichment · Food safety

Introduction

Food contaminated with *Salmonella* is a serious public health concern. Among all foodborne pathogens, *Salmonella* causes the greatest number of annual food safety incidents [1, 2]. The traditional method used to detect *Salmonella* involves multiple steps: preenrichment, selective enrichment, isolation, and biochemical identification for 4–5 days [3]. It does not meet the need for rapid screening to control *Salmonella* contamination in foods, especially in fresh foods or foods with a short shelf life, such as pasteurized milk, fresh meat, and ready-to-eat fruits and vegetables. Because of the serious hazard posed by *Salmonella*, the International Organization for Standardization stipulates "zero tolerance" for *Salmonella* in food [4].

Immunological-based methods are widely used for rapid detection of foodborne pathogens. Recent developments have focused on chemiluminescence immunoassay (CLIA) [5], electrochemical immunosensors [6], surface plasmon resonance immunosensors [7], and cantilever immunosensors [8]. In comparison with these immunosensors, although

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CLIA has equivalent sensitivity [10–10^3 colony-forming units (CFU)/mL] and a slightly longer detection time (about 30 min more), the advantages of good reliability, stability, and compatibility with various targets of in-field tests result in CLIA having good application prospects [3, 9]. In CLIA, a capture antibody is coupled to a solid-phase carrier to capture the target bacteria, and a detection antibody is labeled with an enzyme [e.g., horseradish peroxidase (HRP) or alkaline phosphatase (ALP)] to catalyze the reaction with the chemiluminescent substrate (e.g., luminol or 1,2-dioxetane). A target bacterium-concentrated "sandwich" complex is formed by the capture antibody, target bacterium, and HRP (ALP)–detection antibody. The luminescence intensity is proportional to the enzyme concentration in the reaction, which is directly related to the concentration of the target bacteria, so the target bacteria can be measured qualitatively or quantitatively [10–12]. The HRP–luminol–H_2O_2 system, which is stabler and more sensitive than the ALP–3-(2′-spiroadamantyl)-4-methoxy-4-(3″-phosphoryloxy)-phenyl-1,2-dioxetane system, is used to detect trace targets effectively [13–15]. The analytical sensitivity of CLIA has been increased by adding enhanced chemiluminescent agents to the chemiluminescence system to increase the intensity of luminescence and maintain stability for longer. Studies have shown that the simultaneous use of sodium tetraphenylborate and *p*-phenylphenol synergistically enhances the chemiluminescence of the HRP–luminol–H_2O_2 system and decreases the limit of detection (LOD) [16]. In addition, 4-(1-imidazolyl)phenol has been used as a highly effective enhancer for the HRP–luminol–H_2O_2 chemiluminescence system to increase its detection sensitivity by nearly 50 times [17]. In immunoassay systems, a key factor is the use of solid-phase materials as the carrier to immobilize the capture antibody. Microplates and magnetic beads (MBs) are widely used as solid-phase carriers. Use of polystyrene microplates to detect *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Escherichia coli* O157:H7 in a bacterial suspension resulted in a LOD of 10^4 CFU/mL and a detection time of 3 h [18]. In comparison with microtiter plates, MBs, with their larger surface area, allow the immobilization of a larger number of antibodies and a higher degree of spatial freedom, which results in the acceleration of immune reactions [19–21]. *E. coli* O157:H7 was detected with use of MBs in a chicken carcass rinse with a LOD of 440 CFU/mL and a detection time of 90 min [22]. In addition to reducing the reaction duration and increasing sensitivity, MBs can simplify assays and allow automated detection [23]. The automated chemiluminescence microparticle immunoassay (CMIA) instrument is a relatively mature technology that is used only in various medical tests, such as thyroid tests, metabolism tests, and tumor maker screening [24, 25]. In recent years, we developed a high-throughput CMIA instrument for detection of various targets, including veterinary drug residues, toxins, and pathogens, to meet the need for rapid testing of food samples.

Unlike in blood and urine samples, in food samples of a complex matrix the detection of pathogens requires time-consuming pretreatments, which involve tedious enrichment [4, 26], especially for *Salmonella* detection, for 2 days, so it does not meet the current needs of food safety. Immunomagnetic separation (IMS), which allows specific immunocapture and rapid separation of targets, has been applied in food sample pretreatments and has been adopted in some standard cultivation methods by several countries [27–31] to increase the accuracy of detection of trace target bacteria. Some researchers have attempted to omit the enrichment culture process to shorten the pretreatment time and capture the target bacteria directly in food samples with IMBs. The overall detection time was shortened to 3–6 h with these methods, but their LODs for the target bacteria (more than 10 CFU/25 mL) did not meet the requirement of "zero tolerance" for *Salmonella* detection in food [10, 32–34]. One study showed that the recovery rate of IMBs in 25 mL milk with *Salmonella* at 10 CFU/mL was only 20% [17]. Various characteristics of food samples, including solid particles, excessive salt ions, and excessive acidity or alkalinity, make it difficult to effectively capture and detect trace *Salmonella* in samples, which increases the risk of false negative results in actual applications [23, 35]. The cultivation step in the pretreatment process for pathogen detection is essential and helpful for restoring the viability of pathogens damaged in food processing as well as for inducing proliferation of trace and low-vitality pathogens in samples [26]. The combination of IMS and enrichment culture is used to achieve specific capture and separation of target bacteria in food samples and promotes rapid proliferation of trace target bacteria, thus optimizing the traditional pretreatment process. In many studies, IMS is commonly performed in a small volume (1 mL) after the enrichment process because of the capture performance limits of IMBs [36]. Recently, IMBs with high performance have been used to directly capture trace amounts (10 CFU/mL) of target bacteria from a large sample (25 mL) of a mixed bacteria environment. Modifications of pretreatment methods simplify enrichment procedures and shorten the time required for detection, which can thus satisfy the requirements of various rapid detection methods [37]. However, the detection of 1 CFU of target bacteria per 25 mL (25 g) has not been achieved in food samples.

In this work, we present for the first time a rapid and sensitive method combining an innovative pretreatment strategy and a CMIA to detect trace *Salmonella* in food. The innovative pretreatment strategy, combining in situ enrichment and IMS techniques, was developed specifically to rapidly proliferate *Salmonella* at concentrations less than 10 CFU in 25 mL (25 g) food samples. Subsequently, the CMIA with the enhancer 4-(1-imidazolyl)phenol to increase the luminescence

intensity of the HRP–luminol–H_2O_2 chemiluminescence system was used for sensitive detection. IMBs play two roles: a separation medium to capture *Salmonella* from the food matrix and a solid-phase carrier in the CMIA to allow the direct detection of the pretreatment products. A high-throughput instrument designed especially for food safety applications was used to detect trace *Salmonella* in milk and chicken samples (Fig. 1).

Materials and methods

Reagents and materials

Carboxylic MBs with particle sizes of 180 and 300 nm were provided by Allrun (China), carboxylic MBs with a particle size of 600 nm were provided by Vedo (China), and carboxylic MBs (Dynabeads) with particle sizes of 1 and 2.8 μm were purchased from Invitrogen (Oslo, Norway). Polyclonal *Salmonella* antibodies were purchased from KPL (Gaithersburg, MD, USA). Anti-*Salmonella* group antigen antibodies (HRP marked) were purchased from Abcam (Cambridge, UK). *N*-Hydroxysuccinimide, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide, 2-morpholinoethanesulfonic acid (MES) buffer, Tween 20, luminol, 4-(1-imidazolyl)phenol, citric acid, Na_2HPO_4, and urea–hydrogen peroxide were purchased from J&K Scientific (Beijing, China). Bovine serum albumin was purchased from Biotopped (Shanghai, China). Phosphate-buffered saline (PBS; 25×) (containing NaCl at 100.0 g/L, KCl at 5.0 g/L, Na_2HPO_4 at 28.75 g/L, and KH_2PO_4 at 5.0 g/L), buffered peptone water (BPW), nutrient broth, selenite cystine (SC) medium, Hektoen enteric (HE) agar, and nutrient agar were purchased from Landbridge (China). Pasteurized milk and chicken were purchased from a local supermarket and stored at 4 °C. The milk and chicken samples were confirmed as *Salmonella* negative by the plate culture method.

Bacterial strains and culture conditions

Salmonella Typhimurium (ATCC 14028), *S. enterica* subsp. *enterica* serovar Paratyphi A (ATCC 9150), *S. enterica* subsp. *enterica* serovar Choleraesuis (ATCC 10708), *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (CMCC 63301-5a), *Shigella flexneri* (ATCC 12022), *E. coli* O157:H7 (ATCC 700728), and *Listeria monocytogenes* (ATCC 19115) were purchased from American Type Culture Collection (Manassas, VA, USA). *S. enterica* subsp. *enterica* serovar Enteritidis (CMCC 50041) was purchased from China Medical Culture Collection (Beijing, China), *S. enterica* subsp. *enterica* serovar Anatum (CICC 21498) was purchased from China Center of Industrial Culture Collection, and *Salmonella* Typhimurium (CGMCC 1.1859) was purchased from China General Microbiological Culture Collection.

Salmonella spp., *S. aureus*, *B. cereus*, *S. flexneri*, and *E. coli* O157:H7 strains were cultured at 36 °C overnight in nutrient broth. *L. monocytogenes* was cultured at 30 °C for 24 h. The number of proliferative cells of each bacterial culture was enumerated by plate counting in the nutrient agar.

Preparation of IMBs

Carboxylic MBs of different particle sizes (180 nm, 300 nm, 600 nm, 1 μm, and 2.8 μm) were washed with MES buffer and added to a 1–mL mixture including 10 mg 1–ethyl–3(3–dimethylaminopropyl)carbodiimide and 10 mg *N*-hydroxysuccinimide (dissolved in MES buffer). This was followed by 0.5 h of gentle shaking to activate the beads. The activated MBs were mixed with anti-*Salmonella* polyclonal antibodies at a mass ratio of 100:3, and this was followed by a 2-h incubation at 36 °C with gentle shaking. To block the activation sites of unconjugated antibodies on the MBs, the beads were incubated in a solution containing 1% bovine serum albumin for 0.5 h at 36 °C with shaking.

Capture efficiency of IMBs

IMBs (500 μg) of different particle sizes were separately added to 25-mL samples of pasteurized milk, chicken culture filtrate, and PBS containing Tween 20 (PBST buffer), which each contained 100 CFU *Salmonella*. IMS was performed as follows. The mixture was incubated on a roller for a 1-h antigen–antibody binding reaction at 36 °C, and a magnetic separator (DynaMag-50 magnet) was used to separate the IMBs–bacteria complexes for 0.4 h. The IMBs–bacteria complexes and supernatant were cultured on HE agar plates at 36 °C for 24 h for colony counting.

Next, 200, 500, 750, and 1000 μg of IMBs of different particle sizes were used for IMS in 25 mL pasteurized milk containing 100 CFU *Salmonella*.

The capture efficiency of the IMBs [38] was calculated as follows:

$$\mathrm{CE}\ (\%) = C_1/(C_1 + C_2) \times 100\%,$$

where C_1 and C_2 are the number of *Salmonella* cells captured by the IMBs and in the supernatant, respectively. The experiment was repeated twice to obtain an average and the standard deviation of the measured values.

The binding of IMBs to *Salmonella* was observed under a scanning electron microscope (Merlin, Zeiss, Germany).

IMS for trace *Salmonella*

Milk samples spiked with trace *Salmonella* were prepared by the following procedure. On the basis of the count result (see "Bacterial strains and culture conditions"), the *Salmonella*

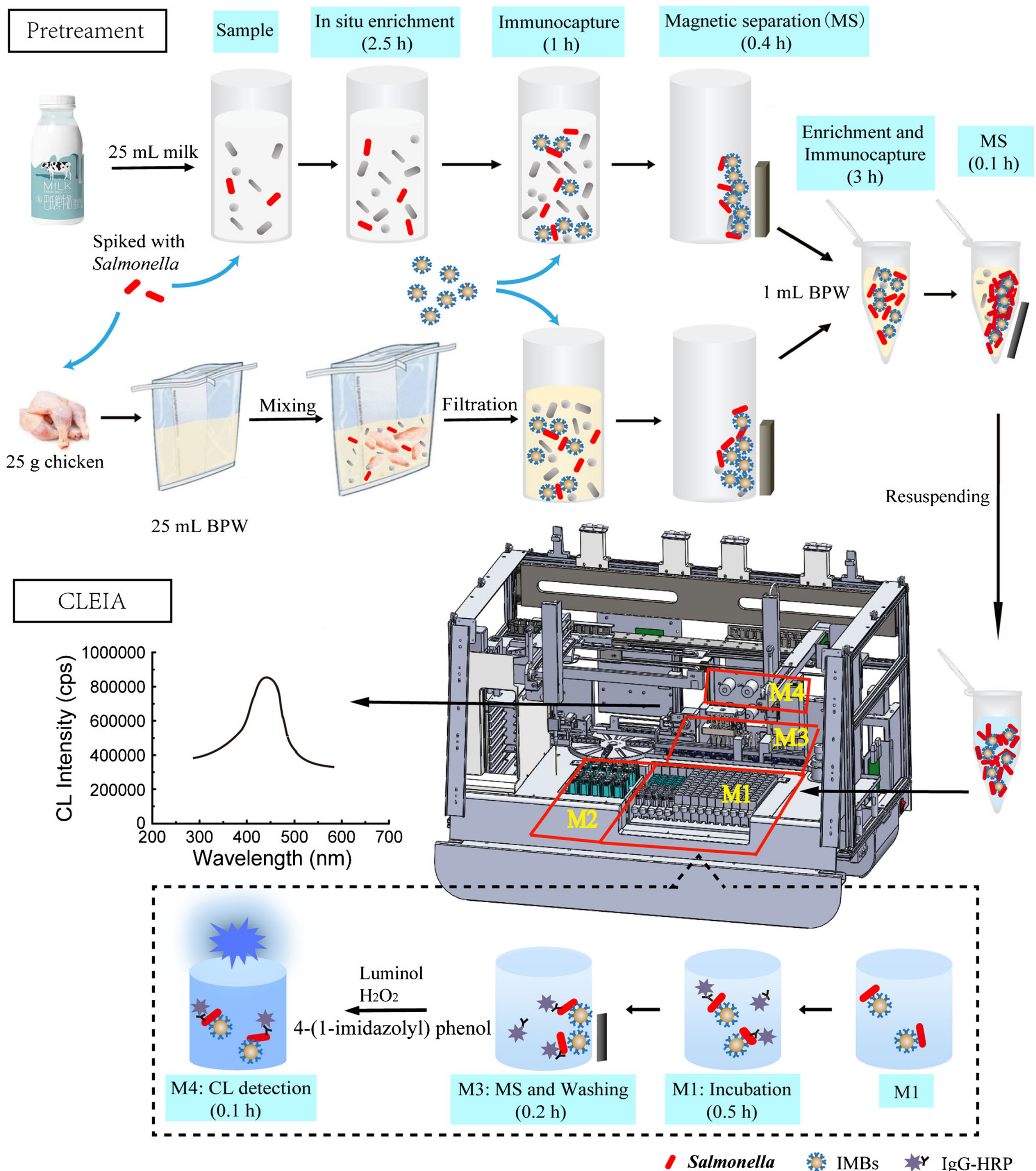

Fig. 1 In situ enrichment–immunomagnetic separation–enrichment–(CLEIA) detection. After preparation, 50 μg of a suspension of *Salmonella*–immunomagnetic bead (IMB) complexes is placed on the microplate of the sample module (M1) of the chemiluminescence immunosensor. The adding module (M2) is responsible for the addition of the detection antibody and the chemiluminescent substrate, and the washing module (M3) is responsible for magnetic separation of the complex and the washing process. Finally, the microplate is raised to the photonic sensor of the chemiluminescence detection module (M4) to detect the luminescence intensity. BPW buffered peptone water, CL chemiluminescence, HRP horseradish peroxidase

Typhimurium culture was serially diluted to about 20 CFU/mL in PBS, and 100, 200, or 300 μL of the suspension was added to 25-mL pasteurized milk samples. To regulate the pH, 1 mL of 25× PBS was added too. IMS was performed on these

samples, and the captured *Salmonella* cells were enumerated as described in "Capture efficiency of IMBs." When there were characteristic colonies of *Salmonella* in the HE agar plate, the result was considered as a successful capture. In addition, enumeration was performed again for the number of *Salmonella* cells that had been added to milk samples. The recovery rate of *Salmonella* by IMS was calculated as follows:

$$P\ (\%) = C_1/C_0 \times 100\%,$$

where C_0 is the average number of *Salmonella* cells added to milk samples and C_1 is the average number of *Salmonella* cells captured by IMS. The experiment was performed with ten spiked milk samples to obtain the average and the standard deviation.

To exclude the samples that had not been spiked with *Salmonella*, for all milk supernatants obtained by IMS detection was performed by a standard cultivation method according to Chinese national standard GB 4789.4-2016 [39]. The supernatant was mixed with 225 mL BPW, and cultured overnight at 36 °C. Then 1 mL of the culture was added to 10 mL SC medium and cultured at 36 °C for 24 h. The culture product was streaked on an HE agar plate to analyze the presence of *Salmonella*. If there was no *Salmonella* in the supernatant or captured by IMS, the sample was not included in the results.

Artificially contaminated chicken samples were prepared as follows. The *Salmonella* suspension mentioned in "Bacterial strains and culture conditions" was refrigerated at 4 °C overnight. The refrigerated fresh chicken sample (25 g) was inoculated with 100 μL suspension. After the addition of 25 mL BPW (containing 0.05% Tween 20), the mixture was incubated at 36 °C for 2.5 h with shaking at 150 rpm. A filtrate was obtained through a piece of nonwoven fabric and transferred to a 50-mL sterile centrifuge tube, after which the filter residue was stored for subsequent detection. IMS was performed on the filtrate, and the captured *Salmonella* cells were enumerated. The rate of recovery of *Salmonella* by IMS was calculated in the same way.

Trace *Salmonella* enrichment in milk and chicken samples

Milk and chicken samples were spiked with the refrigerated 100-μL *Salmonella* suspension as described in "IMS for trace *Salmonella*." These spiked samples were cultured at 36 °C at pH 7.2 with shaking (150 rpm) for 1, 2, 2.5, 3, 3.5, 4, 5, and 6 h. The number of *Salmonella* cells in the chicken culture filtrate or cultured milk was enumerated on an HE agar plate. The experiment was repeated twice.

Pretreatment strategies for trace *Salmonella*

Spiked milk samples were pretreated by the following four strategies (Fig. 2). In strategy 1, a 25-mL milk sample was incubated at 36 °C for 2.5 h with shaking at 150 rpm, followed by IMS for 1.4 h. IMBs–bacteria complexes were resuspended in 1 mL BPW and incubated again for 3 h under the same culture conditions, followed by magnetic separation for 0.1 h. In strategy 2, a 25-mL milk sample was incubated for 5.6 h under the same culture conditions. Then IMS was performed in the milk culture directly. In strategy 3, based on the standard cultivation method, a 25-mL milk sample was added to 225 mL BPW and incubated for 5.6 h under the same culture conditions. Then IMS was performed in 1 mL culture. Strategy 4 was the same as strategy 3 except that IMS was performed in 25 mL culture. The experiment was repeated twice.

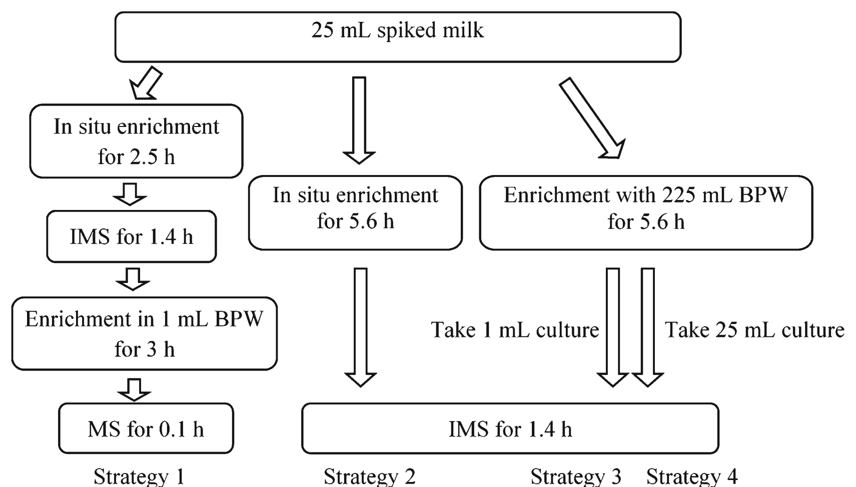

Fig. 2 The processes in the four pretreatment strategies. BPW buffered peptone water, IMS immunomagnetic separation, MS magnetic separation

Optimization of the chemiluminescence detection system

The IMBs–bacteria complexes obtained from strategy 1 were dispersed in 1 mL PBST buffer. Then 5, 15, 50, 150, or 500 μL of the suspension was added to a microtiter plate with an opaque bottom and sidewall, and the microtiter plate was placed into a high-throughput CMIA instrument (HMC-D2, Qinbang, China). The detection procedure started as follows: magnetic separation for 1 min, aspiration of the supernatant, addition 50 μL HRP-labeled antibodies, vortex mixing for 1 min, incubation at 25 °C for 30 min, magnetic separation for 1 min, washing five times with PBST buffer, and incubation with 100 μL of the mixture of chemiluminescence solutions A and B (1:1 ratio). After 3 min of vortex mixing, the luminescence intensity was measured. The entire detection process took approximately 50 min. Chemiluminescence solution A was a mixture of 0.01 M luminol and 0.001 M 4-(1-imidazolyl)phenol (pH 8.8). Chemiluminescence solution B was prepared by addition of 0.1 M citric acid, 0.2 M Na_2HPO_4, and 6.4 mL of 0.75% H_2O_2 to 1 L double-distilled water. Twenty negative samples were tested to obtain the average and standard deviation, and the LOD was set as the average plus three times the standard deviation.

Specificity of the assay

The 25-mL milk samples spiked with 10^4 CFU *E. coli* O157:H7, *B. cereus*, *S. flexneri*, *L. monocytogenes*, or *S. aureus* were pretreated according to strategy 1 (described in "Pretreatment strategies for trace *Salmonella*") and immediately detected by the CMIA as described in "Optimization of the chemiluminescence detection system." The degree of interference [10] was calculated with the following formula:

$$DI = (CL_i - CL_n)/(CL_s - CL_n) \times 100\%,$$

where CL_n, CL_i, and CL_s represent the luminescence intensity of the negative sample, interfering bacteria, and *Salmonella*, respectively.

The applicability of this method to *Salmonella* spp. was tested with strains ATCC 14028, ATCC 9150, CMCC 50041, ATCC 10708, CGMCC 1.1859, and CICC 21498 with a concentration of 1 CFU in 25 mL milk. These experiments above were all repeated twice.

Detection in food samples

Eighty milk and chicken samples were spiked with a refrigerated 50-μL suspension of five strains of *Salmonella* to prepare suspected contaminated samples. All the samples were detected by this method within 8 h via the entire process of in situ enrichment, IMS, enrichment in BPW, IMS again, and CMIA detection. The supernatant of the samples and the rest of the IMBs–bacteria complexes were detected by the standard cultivation method described in "IMS for trace *Salmonella*" to prove the samples were positive or negative.

All samples of pathogenic bacteria and experimental samples contaminated with pathogenic bacteria were sterilized at 121 °C for 45 min before being discarded.

Results and discussion

Capture performance of IMBs

To assess the capture performance of IMBs of different particle sizes for *Salmonella* in food samples, carboxylic MBs of different sizes were coated with *Salmonella* polyclonal antibodies to prepare IMBs under the same reaction conditions. The capture efficiency of 500 μg IMBs (180 nm, 300 nm, 600 nm, 1 μm, and 2.8 μm) for 100 CFU *Salmonella* was examined in 25-mL samples of different liquid matrices (pasteurized milk, chicken culture filtrate, or PBST buffer) (Fig. 3a). The results showed that the capture efficiency of IMBs in PBST buffer was always greater than 75%, and the capture efficiency decreased with increasing particle size. Binding of micron-sized and nano-sized IMBs to *Salmonella* was observed under a scanning electron microscope (Fig. 4). Given

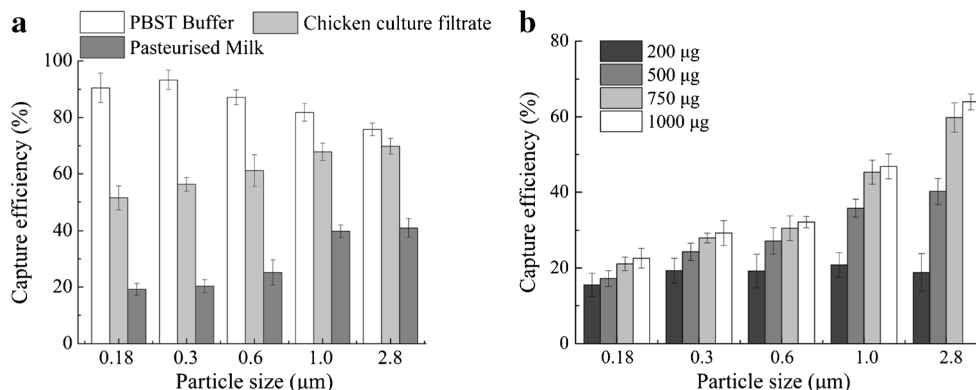

Fig. 3 Capture efficiency in different solutions with 500 μg immunomagnetic beads (**a**) and in milk with different amounts of immunomagnetic beads (**b**). PBST phosphate-buffered saline with Tween 20

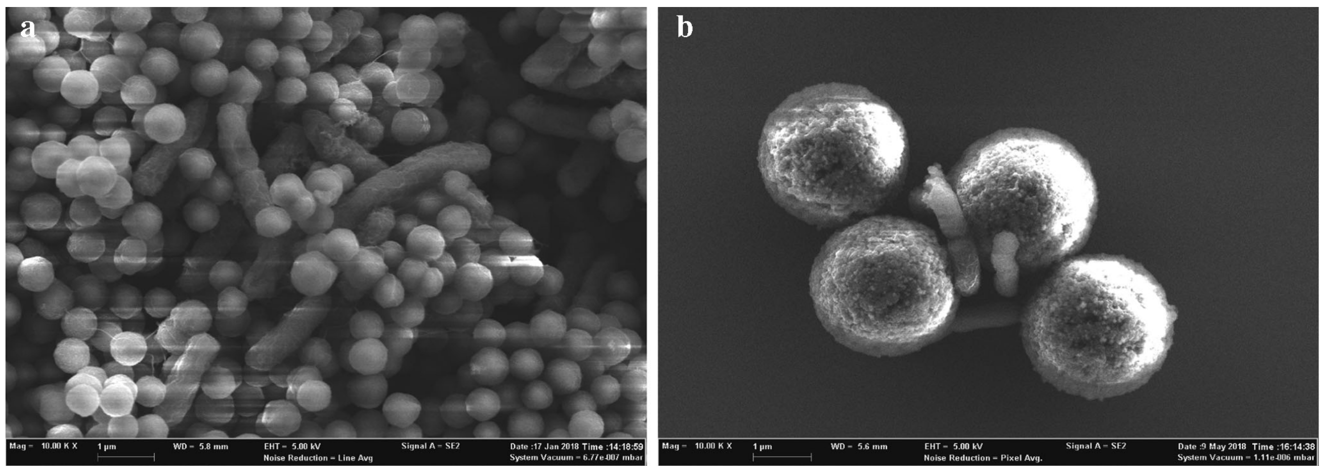

Fig. 4 Scanning electron microscopy micrographs of 0.6-μm immunomagnetic beads (**a**) and 2.8-μm immunomagnetic beads (**b**) capturing *Salmonella*

the same amount of IMBs, a smaller size allowed substantially higher concentrations of particles to interact with the target cells at higher probabilities, and provided larger specific areas to increase ligand packing density. Moreover, excess IMBs shifted the IMBs–bacteria interaction equilibrium toward the depletion of unbound bacterial cells, which manifested itself as increased bacterial recovery [40]. In a simple liquid without interference from the matrix, the capture efficiency of IMBs mainly depended on the amount of particles and the surface antibodies themselves.

In Fig. 3a, the capture efficiency in the chicken culture filtrate and milk is 32.4–60.7% and 19.6–42.1%, respectively, values that were significantly lower than the value in PBST buffer. Moreover, the capture efficiency increased as the particle size increased in the chicken culture filtrate and milk. All the results indicated that the capture performance of IMBs was affected by the food matrix. A liquid food matrix with relatively high viscosity [41], such as milk, increases the resistance of IMBs to migration [42]. When the migration distance during IMS is greatly increased in a large-volume system, and the magnetic recovery performance of IMBs is relatively poor, especially for small beads with large specific surface areas and weak magnetic properties. In the complex food matrix, especially a large-volume system for food safety detection, the magnetic property was the main contributor to the capture performance of IMBs. In previous studies, IMS was applied in small-volume systems or a low-viscosity food matrix, so a different result that capture efficiency decreased as the particle size increased was obtained [38, 40].

As shown in Fig. 3b, the capture efficiency of larger IMBs increased significantly with increase in the amount of IMBs. When 1000 μg of 2.8-μm IMBs was used for immunocapture, the capture efficiency in 25 mL milk reached 63%. For IMBs with good magnetic recovery performance, the capture efficiency could increase through massive use. However, in this study, a larger amount of IMBs would cause a decrease of the sensitivity of the subsequent CMIA detection because of IMBs with a certain absorbance. Because of this, 750 μg of 2.8-μm IMBs with a capture efficiency of 60.7% was used in the next experiment.

IMS and pretreatment strategies for trace *Salmonella*

The feasibility of performing IMS to capture and separate trace *Salmonella* in food samples was studied. IMS was directly performed in 25-mL milk samples spiked with approximately two, four, and six proliferative cells of *Salmonella*, which were enumerated by colony counting, with use of 750 μg of 2.8-μm IMBs (Table 1). In this experiment, a 100% (10/10) capture rate was obtained for the two groups

Table 1 Capture ability of immunomagnetic beads for trace *Salmonella* in a 25-mL milk sample

Salmonella in spiked milk (CFU/25 mL)	Number of successful captures/ number of positive samples	Captured *Salmonella* ± SD (CFU)	Recovery rate (%)[a]	Probability of successful capture (%)[b]
2.1 ± 1.0	8/10	1.8 ± 1.2	86	87.5
3.9 ± 1.1	10/10	3.8 ± 1.7	97	97.5
6.7 ± 1.4	10/10	6.3 ± 2.0	94	99.4

CFU colony-forming units, *SD* standard deviation

[a] Recovery rate = number of captured *Salmonella*/number of *Salmonella* added to milk × 100%.

[b] Calculated on the basis of the *t* distribution of *Salmonella*

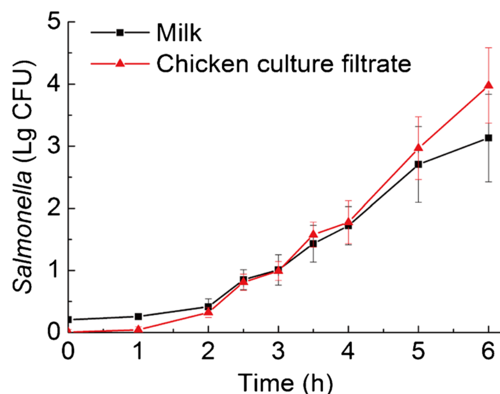

Fig. 5 Proliferation curve for a single *Salmonella* cell in 25 mL milk and chicken culture filtrate. CFU colony-forming units

of samples containing 4 and 6 CFU *Salmonella* (average and standard deviation 3.8 ± 1.7 CFU and 6.3 ± 2.0 CFU, respectively). On the basis of the *t* distribution (with nine degrees of freedom, the Student's *t*-test value at the 95% confidence level was 2.262), the probability of successful capture by IMBs in milk samples containing *Salmonella* at 4 CFU/25 mL was above 97.5%. In addition, according to the binomial distribution, when the capture efficiency of the IMBs was 60.7%, the probability of successful capture was 97.6%. The standard cultivation method for *Salmonella* detection has an accuracy rate of 90–95% [3, 30], which is lower than the rate estimated with the two statistical methods in this work. Therefore, the pretreatment strategy using IMS directly in milk samples containing *Salmonella* at 4 CFU/25 mL or more was found to be feasible.

However, a 20% (2/10) rate of unsuccessful capture was found when IMS was directly used in 25 mL milk containing two proliferative *Salmonella* cells. The recovery rate in each of the three experimental groups (two, four, and six cells) was more than 85%, whereas the capture efficiency was greater than 60.7% (Table 1), which indicated that *Salmonella* utilized the nutrients in milk to proliferate during IMS [43]. Therefore, we designed an in situ enrichment method (in food) before IMS to solve the unsuccessful capture problem that was encountered when 25 mL milk contained less than four *Salmonella* cells. We studied the proliferation of *Salmonella* by simulating refrigerated 25-mL milk samples containing one *Salmonella* cell (Fig. 5). The results showed that 1.7 ± 0.6 CFU *Salmonella* increased to 7.2 ± 1.9 CFU after 2.5 h of in situ enrichment, which was sufficient for IMS [26]. Bacterial cells have been shown to grow and divide even after they are captured on the surfaces of beads [44]. Therefore, the pretreatment strategy for milk was as follows: add buffer to 25 mL of milk for a 2.5-h culture and follow this by immune capture in the milk for 1 h.

For solid food samples such as chicken, it is necessary to transfer trace *Salmonella* to and allow proliferation in a liquid before IMS [34, 38]. The enrichment results are shown in Fig. 5. After 2.5 h of proliferation, the *Salmonella* count in the filtrate increased to 6.5 ± 1.6 CFU. Because the capture efficiency of 750 μg of 2.8-μm IMBs in 25 mL chicken culture filtrate was 74.5%, the probability of successful capture of 3 CFU *Salmonella* was 98.3% on the basis of a binomial distribution. This probability was verified by experiments (i.e., chicken samples infected with 1.6 ± 0.6 CFU *Salmonella* were cultured in BPW for 2.5 h, after which the filtrate was subjected to immune capture for 1 h). The *Salmonella* cells in all experiments were captured successfully, and an average of 8.4 ± 2.6 CFU *Salmonella* was captured. Therefore, the pretreatment strategy for chicken samples was as follows: culture the samples in BPW–0.05% Tween 20 for 2.5 h, and follow this by immune capture from the filtrate for 1 h. In strategy 1 (Table 2), 25 mL milk containing 2.5 ± 1.1 proliferative cells of *Salmonella* was enriched for 2.5 h in situ before IMS for 1 h, followed by enrichment for 3 h in 1 mL BPW; the *Salmonella* count reached 4.73×10^3 CFU. IMS before enrichment of the target bacteria allows specific separation and enrichment of target bacteria from a large number of nontarget bacteria so that the target bacteria become the dominant flora and show faster proliferation in the subsequent enrichment process [37]. Strategy 1 was the most effective

Table 2 The amount and proportion of *Salmonella* obtained by the four pretreatment strategies

Strategy	Spiking with *Salmonella* (CFU/25 mL)	Pretreatment time (h)	Captured *Salmonella* (CFU)	Proportion of *Salmonella* (%)
1	2.5 ± 1.1	7[a]	$(4.73 \pm 1.45) \times 10^3$	11.1 ± 3.7
2	2.5 ± 1.1	7[b]	$(6.04 \pm 1.02) \times 10^2$	0.87 ± 0.31
3	2.5 ± 1.1	7[c]	37.2 ± 12.5	0.21 ± 0.05
4	2.5 ± 1.1	7[d]	$(2.43 \pm 0.92) \times 10^2$	0.09 ± 0.02

[a] In situ enrichment for 2.5 h, immunomagnetic separation for 1.4 h, enrichment for 3 h, and immunomagnetic separation for 0.1 h

[b] In situ enrichment for 5.6 h and immunomagnetic separation for 1.4 h

[c] Enrichment for 5.6 h and immunomagnetic separation (25 mL) for 1.4 h

[d] Enrichment for 5.6 h and immunomagnetic separation (1 mL) for 1.4 h

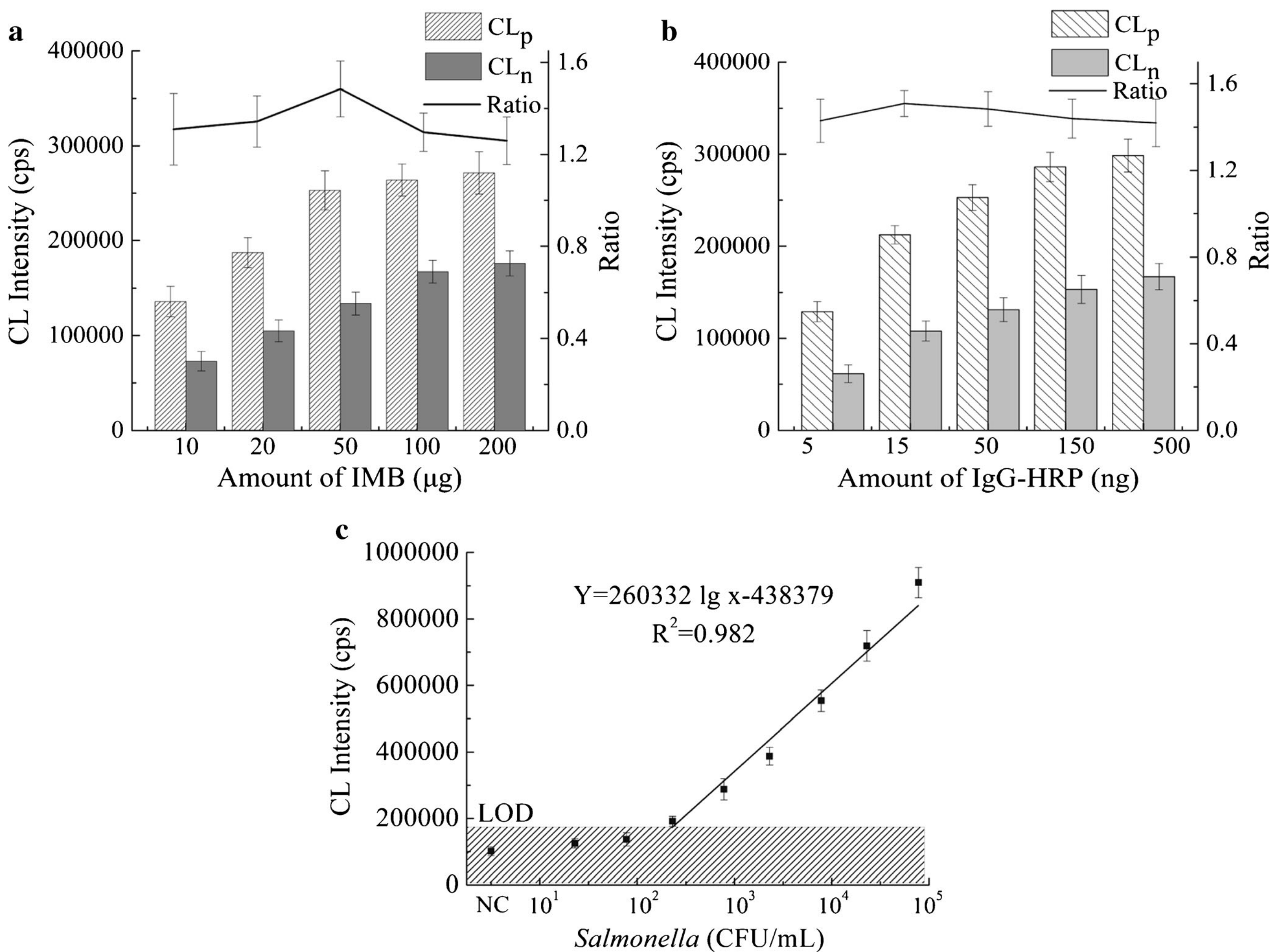

Fig. 6 Optimization for different quantities of immunomagnetic beads (IMBs)–*Salmonella* complexes (**a**) and IgG–horseradish peroxidase (HRP) (**b**), and linear curve for *Salmonella* detection (**c**). The conditions were the optimal conditions. CL_p is the average chemiluminescence of the positive results, CL_n is the average chemiluminescence of the negative results, and "ratio" is CL_p/CL_{LOD}, where LOD is the limit of detection and $CL_{LOD} = CL_n + 3\sigma$. CFU colony-forming units, cps counts per second, NC, negative control, means samples without *Salmonella*.

strategy for obtaining a concentration of target bacteria that exceeded the LOD of the CLIA (10^2–10^3 CFU/mL) [22, 45–47]. Unlike other pretreatment strategies described in previous studies, this strategy, which includes in situ enrichment, can be used to reduce the LOD of fast detection methods to less than 1 CFU.

Optimization of the chemiluminescence system

For more sensitive detection, an HRP-catalyzed luminol–H_2O_2 chemiluminescence system with 4-(1-imidazolyl)phenol as the enhancer was used to detect *Salmonella*, and the quantities of IMBs–*Salmonella* complexes and enzyme-labeled antibodies

Table 3 The degree of interference (DI) in the assay for the interfering bacteria

Bacterial strain	ID no.	CL intensity ± SD ($\times10^5$ cps)	DI (%)
Salmonella Typhimurium	ATCC 14028	31.6 ± 2.0	100
Bacillus cereus	CMCC 63301-5a	1.17 ± 0.09	0.30
Staphylococcus aureus	ATCC 25923	1.40 ± 0.08	1.06
Shigella flexneri	ATCC 12022	1.22 ± 0.07	0.45
Escherichia coli O157:H7	ATCC 21530	1.13 ± 0.10	0.17
Listeria monocytogenes	ATCC 19115	1.25 ± 0.10	0.57%

CL chemiluminescence, *cps* counts per second, *SD* standard deviation

Table 4 Detection of different *Salmonella* serovars

Bacterial strain	ID no.	CL intensity ± SD ($\times10^5$ cps)	LOD ($\times10^5$ cps)
Salmonella Paratyphi A	ATCC 9150	1.92 ± 0.13	≥1.49
Salmonella Enteritidis	CMCC 50041	2.11 ± 0.12	
Salmonella Choleraesuis	ATCC 10708	1.83 ± 0.09	
Salmonella Anatum	CICC 21498	1.79 ± 0.11	
Salmonella Typhimurium	CGMCC 1.1859	1.66 ± 0.12	

CL chemiluminescence, *cps* counts per second, *LOD* limit of detection, *SD* standard deviation

used in the assay were optimized. As shown in Fig. 6a, the luminescence intensity of the positive and negative samples increased as the number of IMBs–bacteria complexes increased, but the ratio of the luminescence of the positive samples to that of the negative samples first increased and then decreased. The highest luminescence ratio was observed when 50 µg of IMB–bacteria complexes was detected with 15 ng of the detection antibody, which indicated that the highest sensitivity was achieved (Fig. 6b). Fifty micrograms of 2.8-µm IMBs was used to detect 0, 23, 78, 230, 780, 2.3×10^3, 7.8×10^3, 2.3×10^4, and 7.8×10^4 CFU *Salmonella* per milliliter by the CMIA. The linear range of this detection method was 2.3×10^2–7.8×10^4 CFU/mL, and the LOD was 230 CFU/mL (Fig. 6c), which was better than the LOD of 1×10^4 CFU/mL reported in a previous study [18]. Similarly, the LODs of the CMIA for *E. coli* O157:H7 and *Legionella pneumophila* are 180 CFU/mL [44] and 2.8×10^3 CFU/mL [45], respectively.

Specificity of the assay

The specificity of the assay was investigated by our assessing its exclusivity for interfering bacteria (non-*Salmonella* bacteria) and inclusivity for *Salmonella* spp. The degree of interference between *Salmonella* and *E. coli* O157:H7, *B. cereus*, *S. flexneri*, *L. monocytogenes*, and *S. aureus* was examined. The luminescence intensity of the interfering bacteria did not exceed the LOD [1.49×10^5 counts per second (cps)], and the degree of interference ranged from 0.17% to 1.06% (Table 3). The degree of interference between *Salmonella* and *B. cereus* or *E. coli* O157:H7 was 0.30% and 0.17%, respectively, whereas values of 3.7% and 1.9%, respectively, were obtained by Xiong et al. [47]. Gehring et al. [48] found that the application of IMS–CMIA technology to the detection of food samples resulted in the problem of excessively high background noise, which interfered with the accuracy of detection and limited the application of the CMIA. To avoid the problem, we utilized the advantage of IMBs to specifically separate the target bacteria and conducted two IMS steps to avoid interference by nontarget bacteria and to increase greatly the proportion of target bacteria in the detection solution, thus reducing the background noise and increasing the accuracy of the CMIA. In addition, we found that the degree of interference between *Salmonella* and *S. aureus* was 1.06%, which was relatively high in comparison with other pairs because protein A on the cell walls of *S. aureus* has high affinity for the Fc fragment of the anti-*Salmonella* antibody [37, 49].

The inclusivity of this method was evaluated with representative strains of different serovars of *Salmonella* [4]. The luminescence intensities of the milk samples containing *Salmonella* (i.e., *Salmonella* Enteritidis, *Salmonella* Paratyphi A, *Salmonella* Choleraesuis, *Salmonella* Anatum, and *Salmonella* Typhimurium) at 1 CFU/25 mL were detected, and all exceeded the LOD (Table 4). The results verified that this method is reliable, with no false negative results, and is applicable to different serovars of *Salmonella*.

Simulated sample detection

Simulated food samples, possibly contaminated with trace *Salmonella*, were studied. Of the 40 milk samples, 22 samples were classified as *Salmonella* positive, whereas

Table 5 Detection in simulated milk and chicken samples possibly contaminated with trace *Salmonella* (data in Table S1)

Sample	Positive			Negative		
	Number	CL intensity ($\times10^5$ cps)	False positive	Number	CL intensity ($\times10^5$ cps)	False negative
Milk	22/40	1.69–5.40	0	18/40	1.01–1.40	0
Chicken	19/40	1.66–4.76	0	21/40	1.01–1.45	0

CL chemiluminescence, *cps* counts per second

Table 6 Summary of detection methods for *Salmonella* in food

Method		Target	Sample	Pretreatment	Limit of detection	Detection time	Reference
Standard cultivation method		*Salmonella* spp.	Any food samples	Preenrichment + selective enrichment + plate separation	1 CFU/25 g	4–5 days	[39]
Immunoassays	CLIA (with polystyrene microtiter plate)	*Salmonella* Typhimurium	Bacterial suspension	–	10^4 CFU/mL	3 h	[50]
	CLIA (enhanced by Au nanoparticles)	*Salmonella* Typhimurium	Bacterial suspension	–	5 CFU/mL	80 min	[33]
Immunoassays combined with concentration methods	CLIA microarray	*Legionella pneumophila*	River water	Filtration and centrifugal ultrafiltration	0.39 CFU/mL before filtration; 2.8×10^3 CFU/mL after filtration	90 min	[45]
	Quantum-dot-based immunoassay	*Salmonella* Typhimurium	Bacterial suspension	IMS	50 CFU/mL	2 h	[51]
	In situ fluorescence immunoassay	*Salmonella* Typhimurium	Chicken	2-h enrichment + IMS	5 CFU/mL	4 h	[52]
Immunoassays combined with cultivation-dependent methods	ELISA	*Salmonella* Typhimurium	Milk	6-h enrichment + phagomagnetic immunoassay	1.4 CFU/25 mL	8.5 h	[53]
	CLIA	*Escherichia coli* O157:H7	Beef	5.5-h enrichment + IMS	10 CFU/g before enrichment; 7.6×10^3 CFU/mL after enrichment	7 h	[48]
	CMIA	5 different *Salmonella* serovars	Milk, chicken	2.5-h in situ enrichment + IMS + 3.5-h enrichment + IMS	1 CFU/25 g	8 h	This work

CFU colony-forming units, *CLIA* chemiluminescence immunoassay, *CMIA* chemiluminescence microparticle immunoassay

19 of the 40 chicken samples were classified as *Salmonella* positive. The chemiluminescence intensities ranged from 1.66 × 10^5 to 5.40 × 10^5 cps, which was higher than the LOD of 1.49 × 10^5 cps (Table 5). By comparison of these results with those obtained with the standard cultivation method, no false negatives or false positives were identified.

Conclusion

This study demonstrates the effectiveness and practicability of a detection method combining a CMIA and IMS pretreatment with cultural enrichment. This method can detect the presence of trace proliferative *Salmonella* in 25 g (25 mL) food within 8 h, which is a prominent advantage compared with other immunoassays (Table 6). In the innovative pretreatment strategy, IMS twice makes *Salmonella* become the dominant flora and shortens the enrichment culture time and reduces interference by other bacteria in the CMIA. Owing to the two-step enrichment culture, trace *Salmonella* such as 1 CFU/25 g can grow in numbers to be detected. As a result, a single proliferative *Salmonella* cell in milk or chicken samples can be enriched to more than 10^3 CFU/mL by a 7-h pretreatment, which meets the LOD requirements of the CMIA. Furthermore, this cultivation process makes the CMIA able to differentiate proliferative *Salmonella* cells from a small amount of nonproliferative cells or cell debris free in the food matrix, whereas sandwich immunoassays are normally not able to distinguish viable and dead bacteria. IMBs play two roles in the method: the separation medium and the solid-phase carrier. We selected 2.8-μm IMBs to obtain excellent magnetic recovery, which contributes to capturing and separating trace *Salmonella* efficiently in a 25-mL sample, and their low light absorbance makes the CMIA highly sensitive. In addition, because of the polyclonal antibody on MBs and the paired antibody-labeled HRP, which can react broadly with different serovars of *Salmonella*, trace *Salmonella* from five different serovars in simulated samples was successfully detected.

Therefore, our method can be used to effectively and rapidly monitor the presence of pathogenic bacteria in small and medium-sized pasture, dairy, and meat processing facilities, as well as in basic-level inspection and quarantine laboratories. This method provides a foundation for the development of a fully automated instrument that include all steps from sample preparation to chemiluminescence detection of foodborne pathogens.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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