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Development of IMBs-qPCR method for detection of foodborne *Salmonella*

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Abstract

Rapid and accurate detection of pathogenic microorganism is critical for food safety. *Salmonella* is one of the common causes of food poisoning. In the present work, polyclonal antibody against the recombinant *PagN* protein was prepared, and coupled with carboxylated magnetic beads to form immunomagnetic beads (IMBs) for capturing *Salmonella*, which was then combined with qPCR technology which used the specific primers of *invA* gene to accurately quantify the number of colonies, thus establishing the IMBs-qPCR method for detection of *Salmonella*. 0.2 mg IMBs could specifically concentrate *Salmonella*, with the stable capturing efficiency of 80%, corresponding to the concentrations of 10^2 - 10^5 CFU/mL. The minimum detection limit concentration was 10^1 CFU/mL. The method was applied for detection and enumeration of *Salmonella* in pork and milk samples, and the capture efficiency of 77.38 and 80.92% were obtained. In summary, the IMBs-qPCR method established herein could effectively detect *Salmonella* with good specificity and sensitivity. The whole detection time was less than 9 h, which laid a foundation for development of a rapid detection kit for foodborne pathogens.

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Introduction

Salmonella is a Gram-negative, non-spore forming, and rod-shaped bacterium, and one of the most common causes of food poisoning in the world for more than 100 years (Bastin *et al.*, 2019). *Salmonella* genus consists of two species namely *S. enterica* and *S. bongori*. The *S. enterica* has a total of six subspecies namely *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI). More than 2,600 serotypes have been found on the basis of their O (somatic) and H (flagellar) antigens for taxonomic classification (Park *et al.*, 2014). Foods such as pork meat, milk, and vegetables are commonly infected with *Salmonella*, which could enter the blood stream when the contaminated foods are swallowed, and then spread through the body (Santander and Curtiss, 2010). The destruction of *Salmonella*, and the release of endotoxin (enterotoxin and cytotoxin) and pro-inflammatory cytokines, provoke damage to cells and tissues (Gilchrist and MacLennan, 2019). Apart from these factors, other virulence factors, including O antigen lipopolysaccharide (LPS), virulence plasmids (containing virulence genes), and flagella and flagellin play an important role in the pathogenesis of *Salmonella* infections (Gut *et al.*, 2018). Depending on whether the infection is acute and limited, or systemic and chronic, *Salmonella* infection causes varying mortality and morbidity. The major diseases are typhoid fever (including paratyphoid fever), invasive non-typhoidal salmonellosis, and non-invasive non-typhoidal salmonellosis (Gut *et al.*, 2018). The detection rates of *Salmonella* isolated from foodborne diseases in China increased continuously in the recent years (Su *et al.*, 2018). In the United States, approximately 1.5 million people die each year from *Salmonella* infections, which is as high as 39% (Velasquez *et al.*, 2018). The detection rate of *Salmonella* in Vietnamese meat foods is about 41.1% (Mather *et al.*, 2018). Therefore, *Salmonella* has become a global public health problem.

The World Health Organization (WHO) has categorised *Salmonella* as a potentially dangerous

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pathogenic microorganism. The testing technologies for *Salmonella* were developing rapidly in the recent decades (Du *et al.*, 2017; Zhai *et al.*, 2019). At present, the detection of *Salmonella* is still primarily based on traditional culture method in most countries, which is time consuming, labour-intensive, and has limitations on testing a large number of samples. Molecular biology methods achieve the detection of pathogens with high accuracy and sensitivity, such as gene chip technology, gene probes, polymerase chain reaction (PCR) (Arunrut *et al.*, 2018), and loop-mediated isothermal amplification (LAMP) (Park *et al.*, 2018), but these methods have higher detection cost and requirements on the professionalism of equipment and operators (Wang *et al.*, 2018). Based on antigen-antibody specific binding capacity, immunological detection technology has been widely used for bacterial detection in the recent years, by mainly targeting the surface antigens of pathogenic bacteria (Zhang *et al.*, 2019). Current common immunoassay-methods include enzyme-linked immunosorbent assay (ELISA), chemiluminescent enzyme immunoassay (CLEIA), immunomagnetic bead separation (IMS), and immunofluorescence labelling technology (IFT) (Zhu *et al.*, 2011; Hyeon *et al.*, 2018). Among them, the immunomagnetic beads (IMBs) are gradually applied to the separation of specific foodborne pathogens due to their strong specificity and ease of separation (Dai *et al.*, 2017). Although a number of immunological detection methods have been reported in recent years, no specific antibody has been demonstrated to cover all the samples for *Salmonella* capturing and detection.

The outer membrane proteins (OMP) commonly participate in the adhesion and invasion of pathogenic bacteria to host cell. These proteins have strong immunogenicity, and can be used as antigens to prepare the vaccine (Futoma-Koloch *et al.*, 2019). *PagN* virulence genes are widely distributed among *Salmonella*, and could be activated by the PhoP system (Lambert and Smith, 2008). The acidic pH and low Mg^{2+} concentration in the external environment induce the expression of *pagN* gene (Nunez-Hernandez *et al.*, 2013). *PagN* protein is located in the outer membrane surface of *Salmonella*, and contains multiple epitopes. It has a β barrel structure that is composed of eight anti-parallel β chains. These β chains contain four extracellular loops, which are essential for the invasion of mammalian cells (Goswami *et al.*, 2018). In addition, *PagN* protein helps *Salmonella* to adhere and invade epithelial cells, and can mediate erythrocyte agglutination (Roche *et al.*, 2018). Therefore, anti-*PagN* antibodies could be used in the capturing and detection of *Salmonella* for different food samples.

In the present work, we developed an IMB separation combined with fluorescent quantitative PCR (IMBs-qPCR) method to detect *Salmonella*. IMBs, the specific anti-*PagN* antibodies coated on magnetic beads that interact with *Salmonella* outer membrane *PagN* protein, could specifically enrich low-concentration *Salmonella* in dairy products. Then, *Salmonella* is enriched and separated from samples by magnetic responsiveness combined with magnetic beads (Li *et al.*, 2019). Combined with qPCR technology to achieve accurate and sensitive detection of *Salmonella* colonies by targeting the *invA* gene, due to the signal strength of fluorophore is proportional to the number of PCR products, thus the initial concentration of unknown templates can be quantitatively analysed (Braun and Methner, 2011). This method has good specificity, high sensitivity, and rapidness. It can be applied for the detection of foodborne pathogens in complex food samples.

Materials and methods

Strain cultivation and extraction of genomic DNA

All bacterial strains used herein are listed in Table 1. All strains were cultured in Luria-Bertani (LB) medium at 37°C with 150 rpm agitation. The bacterial genomic DNA was extracted using the genomic DNA extraction kit following the manufacturer's instruction (Tiangen Biotech Co. Ltd., Beijing, China).

Expression and purification of recombinant PagN protein

We designed *pagN* gene specific primers which contained *Bam*HI and *Xho*I restriction sites (*pagN*-F: 5′-TCGCGGATCCAAAGAAGGGGCCTATATC-3′; and *pagN*-R: 5′-GGTGCTCGAGAAATGCGTAAGTGATGCC-3′) for PCR amplification using bacterial genomic DNA as the template. The 1% agarose gel with ethidium bromide (0.5 μg/mL) was used to analyse the PCR amplification products. The amplified DNA fragments were recovered used TIANgel Purification Kit (Tiangen Biotech Co. Ltd., Beijing, China). Then, the pET28a-*pagN* prokaryotic expression vector was constructed and sequenced.

Table 1. Bacterial strains used in the present work.

Strain	Source	*PagN*	Strain	Source	*PagN*
Salmonella Chittagong	CMCC 50844	+	*Salmonella* Salinatis	CMCC 50830	+
Salmonella Cholerasuis	CMCC 50019	+	*Salmonella* Telaviv	CMCC 50062	+
Salmonella Cholerasuis	CMCC 50020	+	*Salmonella* Typhi	CMCC 50407	+
Salmonella Cholerasuis	CMCC 50306	+	*Salmonella* Typhi	CMCC 50420	+
Salmonella Cholerasuis	CMCC 2146	+	*Salmonella* Typhi	CMCC 50521	+
Salmonella Djakarta	CMCC 50884	+	*Salmonella* Typhi	CMCC 50522	+
Salmonella Gallinarum	CMCC 50770	+	*Salmonella* Typhi	CMCC 50607	+
Salmonella Grumpensis	CMCC 50887	+	*Salmonella* Typhi	CMCC 50618	+
Salmonella Milwaukee	CMCC 50861	+	*Salmonella* Typhi	CMCC 50619	+
Salmonella Paratyphi A	CMCC 50503	+	*Salmonella* Typhi	CMCC 50621	+
Salmonella Poona	CMCC 50136	+	*Salmonella* Typhi	CMCC 50663	+
Staphylococcus aureus	ATCC 25923	-	*Yersinia pseudotuberculosis*	CMCC 53520	-

The pET28a-*PagN* plasmid was extracted and transformed into *E. coli* BL21 (DE3) competent cells, and the positive clones were picked and cultured to logarithmic growth phase. Next, 0.5 mM IPTG was added to induce the expression of recombinant *PagN* protein, which was purified and detected following the method reported previously (Kothari *et al.*, 2006).

Preparation of polyclonal antibodies

The purified recombinant *PagN* protein (1 mg/mL) was used for animal immunity experiments. Two 6-month-old SPF rabbits purchased from Tianjin Laboratory Animal Center were multi-point immunised subcutaneously with 1 mg *PagN* recombinant protein mixed with equal volume of white oil adjuvant. The immunisation consisted of four subcutaneous injections at 9-d interval. Then, the rabbits were euthanised and bled from vein 4 d after the last immunisation. The blood was centrifuged (4°C, 3,200 *g*, for 30 min) to obtain the antiserum.

Anti-PagN antibody purification and concentration determination

The polyclonal antibody was obtained by precipitation with saturated ammonium sulphate from the antiserum of rabbits, and titred using the Bradford method. The concentrations of 0.10, 0.08, 0.06, 0.04, and 0.02 mg/mL of bovine serum albumin (BSA) were prepared to establish the standard curve using Total Protein Quantitative Colorimetric Assay Kit (Lot: E-BC-K168-S) for detecting the protein contents following the manufacturer's instruction (Elabscience Biotech Co. Ltd., Wuhan, China). The standard curve based on the absorbance value and BSA concentration was calculated for determining the antibody concentration. The $OD_{590\ nm}$ value of 10-fold serial dilution (1:10, 1:100, 1:1000, and 1:10000) of anti-*PagN* antibodies against recombinant protein were measured, and the concentration was calculated using the standard curve. Using diluent (PBS) as the negative control, no less than three biological replicates were made for each experiment.

Western blot

The purified recombinant *PagN* protein was separated by SDS-PAGE, and transferred to the PVDF membrane. Then, the anti-*PagN* polyclonal antibody was diluted at 1:300 for incubation, followed by washing with TBST buffer for three times at 30-min interval. Next, incubation with HRP goat anti-rabbit IgG antibody (CW0103S, CWBIO Corp, Beijing, China) at room temperature for 1 h was done as described previously (Wang *et al.*, 2018). Finally, immunodetection was completed by using ECL Western blotting kit (Pierce) and chemiluminescence apparatus (Gel Doc XR+ Imaging System, BIO-RAD, USA).

Indirect ELISA

Briefly, 5 μg purified recombinant *PagN* protein diluted with carbonate buffer (pH 9.6) was added into 96-well plate at 100 μL/well, and incubated overnight at 4°C. Then, the plate was washed with 0.1% Tween 20 PBS buffer for three times, and blocked with 1% BSA-PBST buffer at 37°C for 30 min. The serial dilutions of toxin antibody or negative rabbit serum (negative control) were added at 100 μL/well. The HRP-labelled goat-anti-rabbit IgG conjugate was added at 100 μL/well, and incubated at 37°C for 30 min. Then, 100 μL of *O*-

phenylenediamine (OPD) substrate colouring solution (Solarbio Sci & Tech Co. Ltd., Beijing, China) were added to each well, and incubated at 37°C for 10 min. Finally, the stop solution was added to stop the activation. The value of OD_{490nm} was measured using a microplate reader (Bio-Rad, USA). Three biological replicates were performed for each experiment.

The antibody titre of rabbit anti-*PagN* was calculated by formula: P/N = (OD value of sample – OD value of blank slot) / (OD value of negative serum - OD value of blank slot). The maximum serum dilution ratio is defined as its ELISA titre as the P/N value was 2.1.

Preparation of immunomagnetic beads

Affimag PSC magnetic beads (mean diameter of 1 - 2 μm, 10 mg/mL) were purchased from BaseLine Tech Co. Ltd. (Tianjin, China). The carboxylated magnetic beads (1 mg) were washed with 700 μL of 0.01 M phosphate buffer saline (PBS, pH 6.0) containing Tween 20 (0.05% v/v) for three times, and then resuspended in 600 μL of freshly made 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC, 5 mg/mL) and *N*-hydroxysuccinimide (NHS, 5 mg/mL) solution in 0.01 M PBST (pH 6.0) for activation in orbital shaker at 37°C for 30 min. After activation, the magnetic beads were washed three times with 0.01 M PBST (pH 7.4), and resuspended in PBST buffer (pH 7.4). Then, purified anti-*PagN* antibody was added to fully couple to form the IMBs. Next, 30 μL anti-*PagN* antibody with concentration of 14.686 mg/mL was added to couple with 1 mg of carboxylated magnetic beads that were combined for 1, 2, 3, 4, and 6 h and shaken at 37°C. Then, the magnetic beads were enriched using magnet device following the manufacture's instruction (BaseLine Tech Co. Ltd., Tianjin, China), and the antibody concentration in the supernatant was measured at 1, 2, 3, 4, and 6 h, respectively. The optimal time for coupling the antibody to the magnetic beads was calculated. Finally, 1% BSA solution was added to block the unreacted carboxyl group.

Optimisation of immunomagnetic bead capture capability

The prepared IMBs with different dosages (0.0125, 0.025, 0.05, 0.075, 0.1, and 0.2 mg) were used to capture *Salmonella*. The bacteria were cultivated to 10^6 CFU/mL, and diluted to 10^1 CFU/mL in a 10-fold gradient. The optimal dosage of IMBs to capture *Salmonella* was 0.2 mg at the order of 10^1 - 10^6 CFU/mL. Three parallels were set for each sample. The number of *Salmonella* before and after capturing was counted respectively by the method of plate counting, and used to calculate the capture efficiency of IMBs.

Scanning electron microscope

The glass slide was cut into a 1 × 1 cm square with a glass knife, and soaked in 75% ethanol solution overnight. The next day, IMBs were prepared for capturing *Salmonella* Typhi CMCC 50619 with 10^4 CFU/mL as described earlier. After capturing, the compound was absorbed by the magnetic frame. Afterwards, the IMBs-*Salmonella* complex was washed twice with sterile distilled water, which could remove the salt crystals in PBS to avoid the large crystals observed under the scanning electron microscope. Then, the IMBs-*Salmonella* conjunctures on the glass slide were dried in a 37°C incubator. After drying, the samples were plated with gold, followed by observation and imaging under field emission scanning electron microscope Nanosem 430. SEM specifications: acceleration voltage, 0.1 - 30 KV; magnification, 200,000 - 800,000×; and resolution, 1 nm.

Quantification of Salmonella by qPCR

The *Salmonella* stock solution, which was cultivated for 12 h at 37°C, was diluted to 10^0 - 10^7 CFU/mL to extract genomic DNA as the template for fluorescent quantitative PCR (qPCR) detection. Due to the significant specificity of *Salmonella invA* gene, it could be used to design specific qPCR primers for *Salmonella* detection (qPCR-*invA*-F: 5`-AAAGGAACGGGTTGCTGTAA-3`; and qPCR-*invA*-R: 5`-TATCAGGACGTTTTTTCCGC-3`; The bands size of qPCR amplification was 200 bp). Following the fluorescence PCR kit's instruction, 20 μL of reaction system was prepared with TransStart Top Green qPCR Super Mix (TransGen Biotech, Beijing, China), and performed using ABI 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). The qPCR amplification program for the *invA* gene was as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 15 s, 56°C for 30 s, and 72°C for 40 s.

A specific interference test was carried out on the *invA* detection primers. *Y. enterocolitica* (CMCC 55075), *E. coli* DH5α, and *S. aureus* (ATCC 25923)

were selected as interfering strains, and genomic DNA was extracted from *Salmonella* mixed with background strains for qPCR detection. The IMBs capture and qPCR experimental conditions (including temperature and time, *etc*.) of the entire interference experiment were consistent with the *Salmonella* detection experimental conditions. Three biological replications were performed for each experimental group. After the program was completed, the C_T values shown as the ordinate represented the average of three samples for each qPCR group. The number of *Salmonella* colony corresponding to the C_T value was used as the abscissa. The standard curve was drawn using the Origin software.

IMBs-qPCR of Salmonella in food samples

To ensure the uniformity of the experiment, whole milk (Beijing Sunyuan Food Co. Ltd., Beijing, China) and fresh pork (Gongs Group, Shandong, China) of the same brand were purchased from a local store (WuMart Supermarket) for rapid detection of *Salmonella*. Food samples were sterilised by freezing (-80°C) and ultraviolet irradiation. The standard plate culture method determined that the food samples used were negative for *Salmonella*. Firstly, the mixture of 20 mL of whole milk or 20 g of pork meat with 180 mL of buffered peptone water (BPW) medium was homogenised. After that, 10^1 CFU/mL *Salmonella* bacterial solution (1 mL) was added into the mixture. Next, they were kept at 4°C for 48 h to allow bacteria to better adapt to the new environment. Afterwards, the complex was shaken with 150 rpm at 37°C for 4.5 h to increase the concentration of bacteria. After enrichment, 1 mL of sample was collected for capturing target bacteria with 0.2 mg of IMBs for 30 min. The number of *Salmonella* after culturing for 5 h in 1 mL of BPW medium was the total number of S*almonella*. Genomic DNA was extracted using captured IMBs-bacteria complex for qPCR detection. The capture efficiency was calculated by plate counting method, and three replicates were performed for each sample.

Results

Anti-recombinant PagN polyclonal antibody with good specificity

To determine the broad-spectrum of selected *PagN* virulence genes, we extracted genomic DNA from 22 strains of *Salmonella* which belonged to different serotypes and two other bacterial strains (*Staphylococcus aureus* ATCC 25923 and *Yersinia pseudotuberculosis* CMCC 53520). The results confirmed that the virulence gene was generally present in all the strains of *Salmonella*, with a size of about 720 bp by PCR amplification through *pagN* gene-specific primers, but absent in *S. aureus* ATCC 25923 and *Y. pseudotuberculosis* CMCC 53520 (Table 1). Combined with the analysis of DNA and protein sequence, *pagN* could be a specific detection gene for *Salmonella*.

After constructing the prokaryotic expression vector of pET28a-*pagN*, the recombinant protein with 6× histidine tag was expressed and purified. The molecular weight of recombinant *PagN* protein was about 28 kDa, as measured by SDS-PAGE (Figure 1A). Subsequently, the purified protein was used to immunise rabbits to prepare *PagN* antiserum that was used to obtain the polyclonal antibodies. The antibody titre of specific anti-*PagN*-polyclonal antiserum was calculated. This was determined as 1:3200 (Figure 1B). Simultaneously, Western blot results showed that the antibody could specifically recognise *PagN* recombinant protein, and the *PagN* protein in natural *Salmonella* strains (Figure 1C), thus confirming the specificity and effectiveness of the prepared anti-*PagN* polyclonal antibody.

Optimisation of capturing scheme of immunomagnetic beads for Salmonella

To optimise the capture efficiency of carboxylated magnetic beads for anti-*PagN* antibody, we explored the optimal time for carboxylated magnetic beads conjugated antibody. The results showed that when the coupling time was 4 h, the amount of antibody on magnetic beads reached maximum of 73.82 μg (Figure 2A). In order to explore the optimal amount of IMBs to capture *Salmonella*, various amounts of IMBs (0.0125, 0.025, 0.05, 0.075, 0.1, and 0.2 mg) were used to capture *Salmonella*, and the concentration was 10^5 CFU/mL. Plate culture method was used to calculate the IMBs capture efficiency. The results showed that with the increase in the amount of IMBs, the capture efficiency also increased significantly, but the upward trend slowed down, where 75.70, 73.83, and 79.44% capture efficiency were found for 0.075, 0.1, and 0.2 mg IMBs, respectively. So, it was also determined that the 0.2 mg IMBs had the highest capture efficiency (Figure 2B).

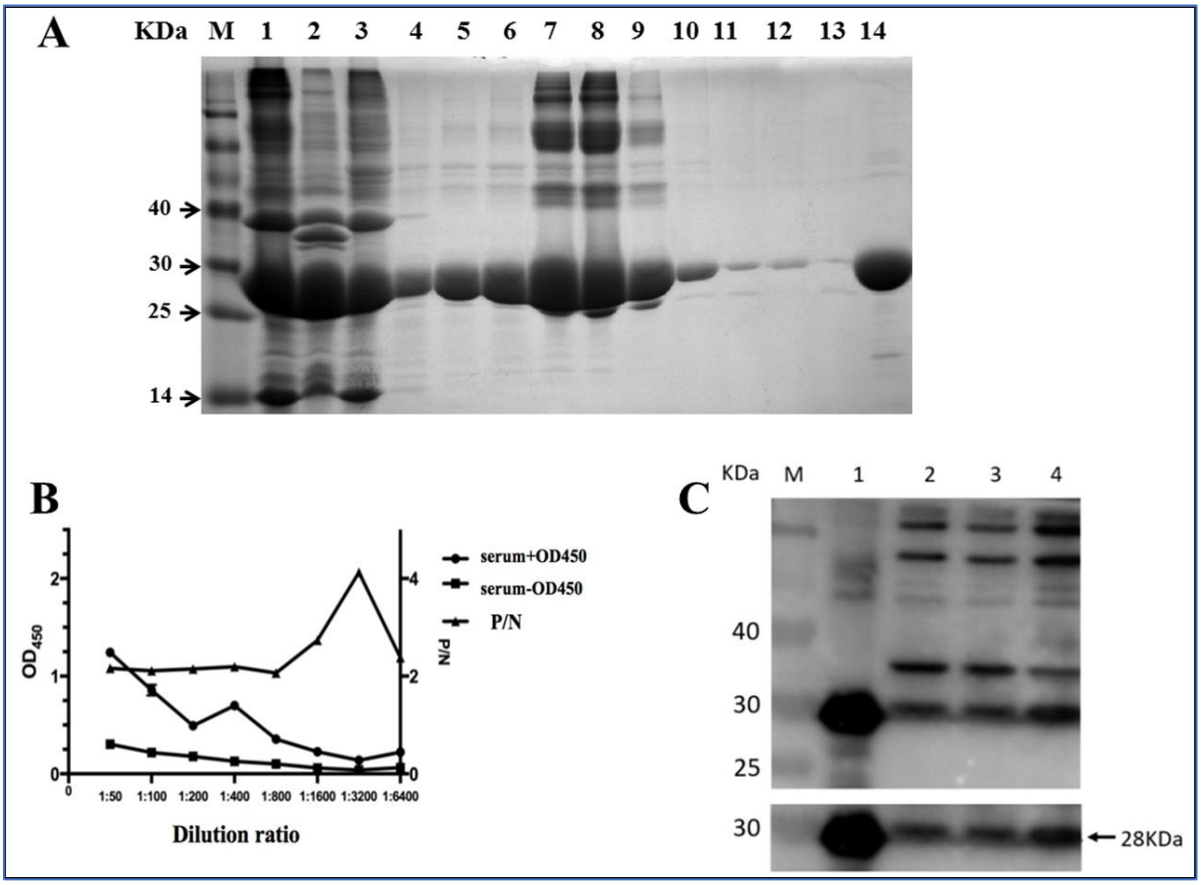

Figure 1. The prepared *PagN* polyclonal antibody has high specificity. **(A)** The recombinant *PagN* protein was analysed by SDS-page after induced with IPTG and purified from *E. coli* BL21 (DE 3). Lane M, molecular mass markers (Blue Plus II Protein Marker, TransGen Biotech, Beijing, China); lane 1, bacterial supernatant after dissolving with 8 M urea; lane 2, bacterial precipitation after dissolving with 8 M urea; lane 3, penetrating solution; lane 4, washing results with 20 mM imidazole solution; lane 5 - 12, 250 mM imidazole and 8 M urea results of protein purification; lane 13, supernatant after dialysis; lane 14, precipitation after dialysis. **(B)** ELISA titre determination of anti-*PagN* polyclonal antibody. P/N = (OD value of sample – OD value of blank slot) / (OD value of negative serum – OD value of blank slot). **(C)** The polyclonal antibody against *PagN* protein reacted with natural *PagN* protein from different *Salmonella* isolates by western blot. Lane M, molecular mass markers (Blue Plus II Protein Marker, TransGen Biotech, Beijing, China); lane 1, PagN recombinant protein purified from *E. coli* BL21 (DE 3); lanes 2 - 4, two different serotypes of *Salmonella* and one *Staphylococcus* strain (*S.* Typhi CMCC 50619, *S.* Typhi CMCC 50503, and *S. aureus* ATCC 25923, respectively).

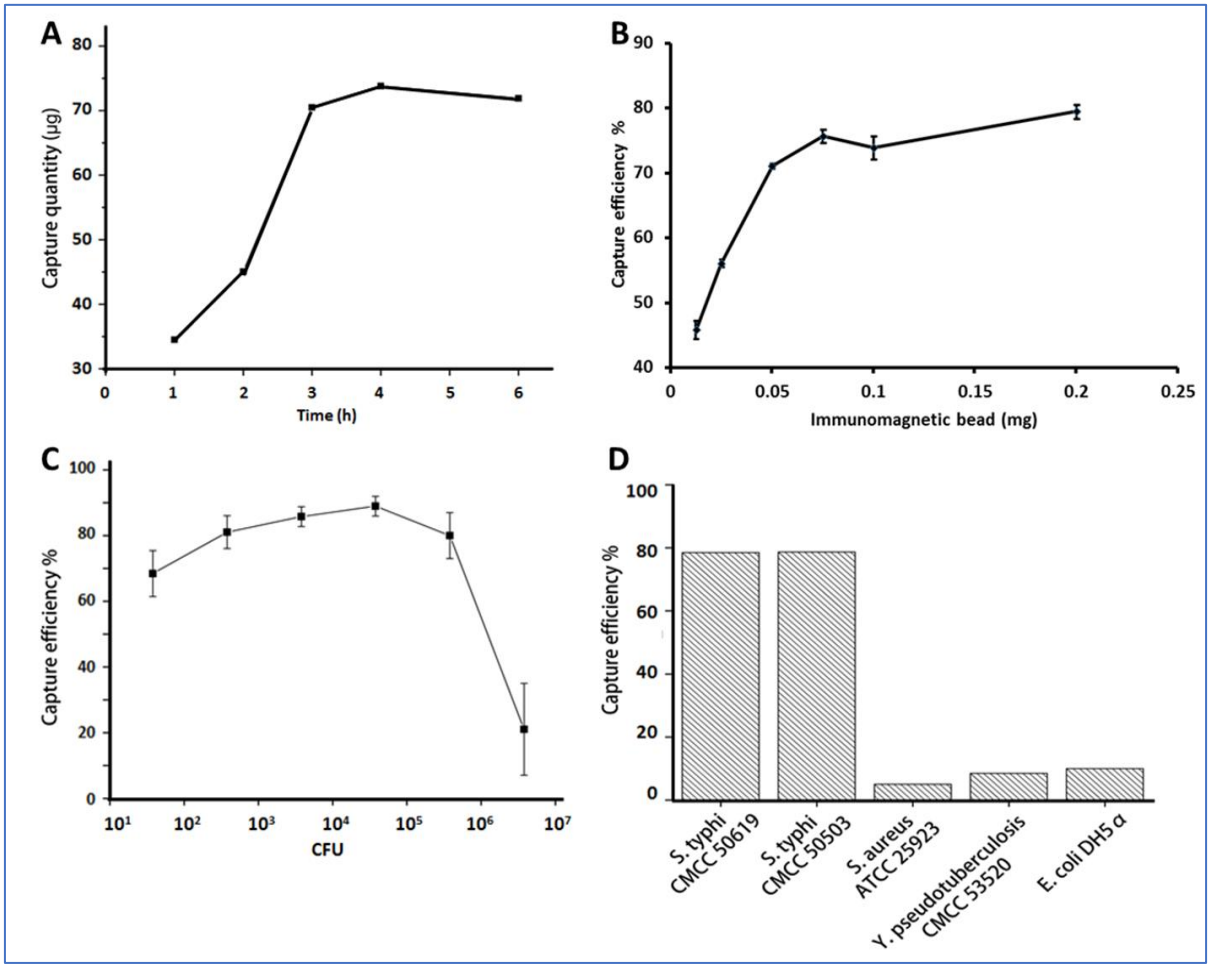

Figure 2. Optimisation scheme of IMBs to capture *Salmonella*. **(A)** Determination of the optimal time for magnetic beads conjugated antibody. *x*-axis: time (h), *y*-axis: capture quantity (μg). **(B)** Capture efficiency for the different amounts of IMBs. *x*-axis: IMBs (mg), *y*-axis: capture efficiency (%) (three biological replicates per group). **(C)** Capture efficiency of *Salmonella* at different concentrations by IMBs. *x*-axis: log CFU; *y*-axis: capture efficiency (%) (three biological replicates per group). **(D)** Specific evaluation of IMBs for capture efficiency of different bacteria. From left to right: *S.* Typhi CMCC 50619, *S.* Typhi CMCC 50503, *S. aureus* ATCC 25923, *Y. enterocolitica* CMCC 53520, and *E. coli* DH5α, respectively.

Next, 0.2 mg IMBs were used to capture (3.8 ± 0.2) × 10^1 - 10^6 CFU/mL orders of *Salmonella*. The results showed that the capture efficiency could reach 81.05, 85.78, 88.94, and 80.00% for 10^2 - 10^5 CFU/mL *Salmonella*, respectively. For 10^1 CFU/mL *Salmonella*, the capture efficiency was 68.42 ± 7%, lower than 70% (Figure 2C and Table 2). The reason for low capture efficiency may be that the colonies content was too low to collide with IMBs. And when *Salmonella* was more than 10^5 CFU/mL, the reason for low capture efficiency may be that the amount of IMBs has reached saturation, so it was no longer possible to capture excess *Salmonella*. The above experimental results showed that the optimal time for carboxylated magnetic beads conjugated anti-*PagN* polyclonal antibody was 4 h at 37°C. The capture efficiency of IMBs (0.2 mg) for *Salmonella* on the orders of 10^2 - 10^5 CFU/mL could reach more than 80%. Therefore, the follow-up detection of *Salmonella* in food samples was referred to this scheme.

Table 2. Capture efficiency of IMBs-qPCR for different orders of *Salmonella*.

IMBs (mg)	a (CFU/mL)	b (CFU/mL)	CE (%)
0.2	(3.8 ± 0.2) × 10^1	1.2 ± 0.3 × 10^1	68.42 ± 7
0.2	(3.8 ± 0.2) × 10^2	7.2 ± 0.2 × 10^1	81.05 ± 5
0.2	(3.8 ± 0.2) × 10^3	5.4 ± 0.1 × 10^2	85.78 ± 3
0.2	(3.8 ± 0.2) × 10^4	4.2 ± 0.1 × 10^3	88.94 ± 3
0.2	(3.8 ± 0.2) × 10^5	7.6 ± 0.3 × 10^4	80.00 ± 7
0.2	(3.8 ± 0.2) × 10^6	3.0 ± 0.6 × 10^6	21.05 ± 14

a: total number of *Salmonella* in each sample before IMBs capture. b: total number of *Salmonella* in the supernatant after IMBs capture. CE: capture efficiency with IMBs. CE1 (%) = (a – by) / a × 100%. Three replications were performed for each group.

Specific evaluation of capture efficiency for different bacteria

To analyse the specificity of IMBs captured bacteria, 0.2 mg of IMBs were used for capturing the target bacteria from the solution of mixed *S.* Typhi CMCC 50619, *S.* Paratyphi CMCC 50503, *Staphylococcus aureus* ATCC 25923, *Yersinia pseudotuberculosis* CMCC 53520, and *E. coli* DH5α, at the concentrations of 10^5 CFU/mL. The capture efficiency was calculated using plate count method. Results showed that the capture efficiency of IMBs for other common strains was much lower than that of *Salmonella* (Figure 2D), which indicated that the prepared IMBs could specifically capture *Salmonella*.

IMBs-Salmonella complex observed by scanning electron microscopy

To visually determine whether IMBs could capture *Salmonella*, the samples were observed by the field emission scanning electron microscope Nanosem 430. The *Salmonella* colonies were clearly observed under the electron microscope (Figure 3A). The surface of magnetic beads was uneven due to the conjugation of polyclonal antibodies; they were easy to aggregate because of the adhesion among the beads (Figure 3B), and the combination of IMBs-bacteria complex was observed (Figure 3C). These results further proved the feasibility of the detection method established in the present work.

Specificity evaluation of IMBs-qPCR

Based on the linear relationship between the C_T value of fluorescent qPCR amplification products and the logarithm of the initial concentration of genomic DNA template, the standard curve (y = -3.61x + 40.40, R^2 = 0.9979) for qPCR detection of *Salmonella* was established using the specific primers of the invasion gene *ivnA* that is unique to the genus *Salmonella* (Figure 4A). The detection technology failed to detect the C_T value when *Salmonella* sample was at 1.31 × 10^0 CFU/mL. The evaluation of the stability of standard curve was repeated four times. The interference test was carried out using different bacterial genomic DNA as templates with *invA* primers in qPCR assay. The results showed that there was no significant difference in C_T value among the group of *Salmonella*, mixed with or without different interfering bacterial strains (Figure 4B). The presence

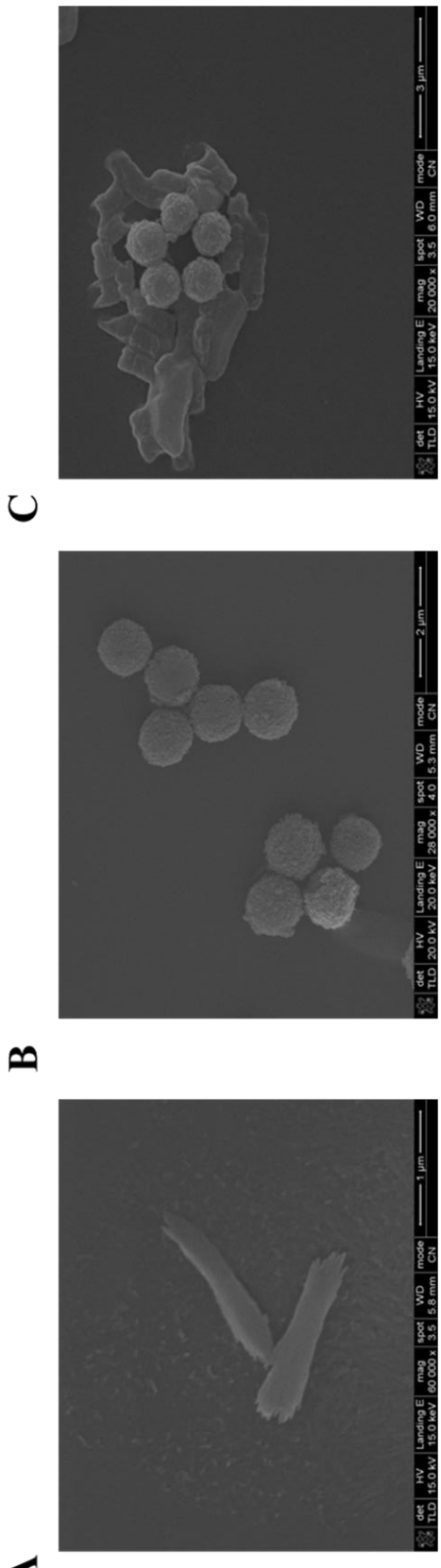

Figure 3. Scanning electron microscope observation of IMBs to capture *Salmonella*. **(A)** *Salmonella* Typhi CMCC 50619, **(B)** immunomagnetic beads (IMBs), and **(C)** capture of *Salmonella* by IMBs.

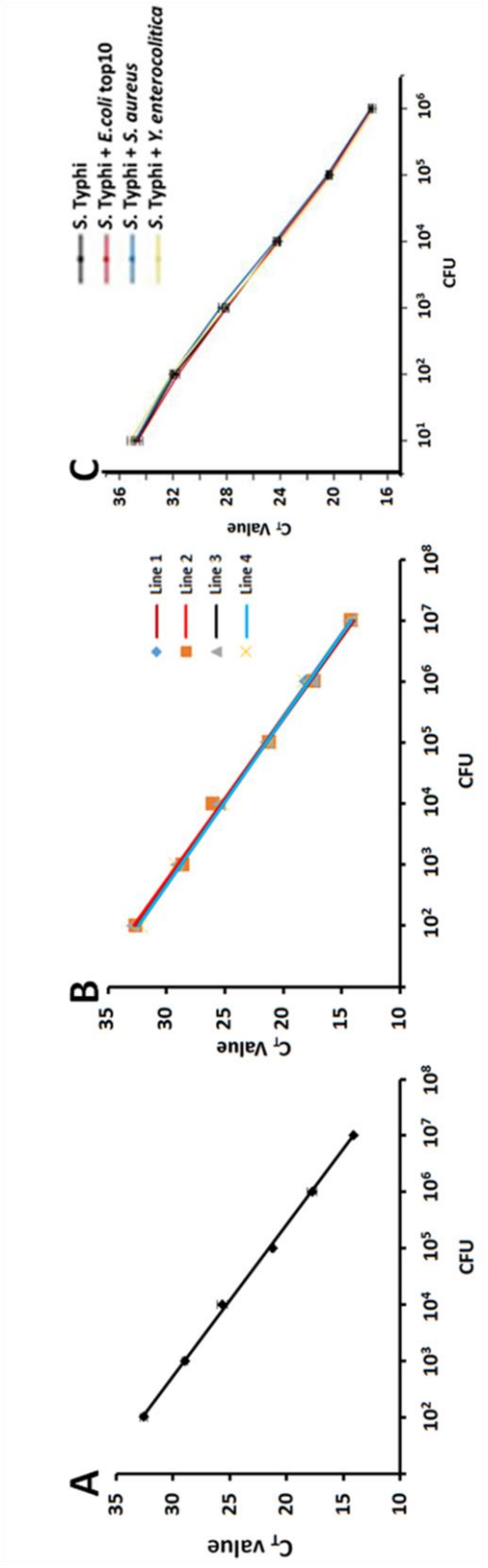

Figure 4. Evaluation of the specificity and minimum detection limit of IMBs-qPCR. **(A)** Establishment of qPCR standard curve and stability evaluation which was used for accurate number of *Salmonella* colonies. *x*-axis: log CFU, *y*-axis: C_T (cycle threshold) value. The evaluation of the stability of standard curve was repeated four times. **(B)** Specific interference test of *Salmonella* detection *invA* primers to evaluate the possibility of *invA* gene non-specific detection of other bacteria. *Yersinia enterocolitica* (CMCC 55075), *Escherichia coli* DH5α, and *Staphylococcus aureus* (ATCC 25923) were selected as the interfering strains, mixed with *Salmonella* and diluted genomic DNA for qPCR detection. Standard curve of the C_T value. Line 1: *S.* Typhi + *E. coli* top 10; line 2: *S.* Typhi; line 3: *S.* Typhi + *S. aureus*; and line 4: *S.* Typhi + *Y. enterocolitica*. **(C)** Amplification curve of qPCR interference test. Black line: *S.* Typhi; red line: *S.* Typhi + *E. coli* top 10; blue line: *S.* Typhi + *S. aureus*; yellow line: *S.* Typhi + *Y. enterocolitica*.

of background strains could not interfere with the qPCR amplification using *Salmonella invA* gene primers. There was only a single peak at the dissolution curve, and no non-specific binding occurred (Figure 4C). In summary, the selected *invA* primers had good specificity for qPCR detection technology of *Salmonella*.

Application of IMBs-qPCR method in food samples and determination of detection limit

We found no *Salmonella* in fresh milk or pork samples using traditional culture method. And it was hard for us to get the real food samples contaminated with *Salmonella.* Therefore, fresh milk and pork were artificially contaminated with 10^1 CFU/mL *Salmonella* for pre-enrichment. After 5 h incubation, *Salmonella* reached 10^4 - 10^5 CFU/mL. Then, the IMBs-qPCR method was used to detect the number of *Salmonella* in food samples. The results showed that the capture efficiency by IMBs in pork and milk samples could reach 71.43 and 80.92%, as detected by qPCR method. The number of colonies in the supernatant after IMBs capture showed the efficiency of 77.38 and 80.15% for pork and milk, respectively, as detected by traditional culture method (Table 3). The results showed that the capture efficiency by IMBs in pork or milk samples had good reproducibility. The whole detection process could be finished within 9 h. There was no impurity peak of qPCR, and the correlation coefficient was fitting well. The detection limit of *Salmonella* using IMBs-qPCR rapid detection system established in the present work was 10^1 CFU/mL.

Table 3. Capture efficiency of IMBs-qPCR in food samples.

Sample	a (CFU/mL)	b (CFU/mL)	c (CFU/mL)	CE1 (%)	CE2 (%)	*r*
1 mL milk	1.31×10^5	2.6×10^4	1.06×10^5	80.15	80.92	1.01
1 mL pork solution	8.4×10^4	2.4×10^4	6.5×10^4	71.43	77.38	1.08

A: total number of *Salmonella* in 1 mL of BPW medium after 5 h enrichment as detected by plate culture method. B: total number of *Salmonella* in supernatant after IMBs capture as detected by plate culture method. C: number of *Salmonella* in BPW medium after 5 h enrichment as detected by IMBs-qPCR method. CE: capture efficiency with IMBs. CE1 (%) = (a – b) / a × 100% ; CE2 (%) = c /a × 100%. *r*: correlation coefficient. *r* = CE2 (%) / CE1 (%). Three replications were performed for each group.

Discussion

Salmonella, as the main pathogen causing food poisoning, has become a global public health problem. It is particularly important to develop and establish detection techniques that comply with national standard methods (Piltch-Loeb *et al.*, 2018). The present work aimed to establish a set of accurate, effective, and economical IMBs-qPCR method, which was mainly based on magnetic beads-antibody (anti-*PagN* recombinant protein, IMBs) conjugating and qPCR detection (specific *invA* gene primers). This method could specifically identify *Salmonella* serotypes and avoid the possibility of detecting other interfering bacterial strains (Mohammed *et al.*, 2017). In addition, the number of colonies can be captured accurately and quantitatively within 9 h in the present work. The minimum detection limit was 10^1 CFU/mL. Due to the absent of IMBs for enrichment of the bacteria, one multiplex PCR method showed the detection limit was only 10^3 CFU/mL for several common bacterial pathogens, including *Salmonella* in pure culture (Arunrut *et al.*, 2018). The detection method of nano- and micro-IMBs with PCR for *Salmonella* in chicken could be completed within 8 h (Dai *et al.*, 2017). The method developed herein could solve the problems of low sensitivity, long time, and poor specificity in the detection of *Salmonella* (Yin *et al.*, 2016).

The virulence genes of *Salmonella* are the main cause of their pathogenicity. In general, the outer membrane proteins encoded by bacterial unique virulence genes have high immunogenicity; so, we set standards to screen *Salmonella* genes for preparation of IMBs and detection of this bacterium (McIntosh *et al.*, 2017). Firstly, the virulence genes that are related to the pathogenicity and have species specificity for *Salmonella* were chosen. Secondly, the outer membrane protein genes were the potential candidates that encode OMP located on the cell surface of *Salmonella* involved in the antigen-antibody specific binding during *Salmonella* capturing with prepared IMBs. Finally, the genes on the chromosome that could keep hereditary stability

between different bacterial isolates were required. Furthermore, the virulence antigens with larger molecular weight and more epitopes are beneficial for improving capture efficiency. Based on these selection criteria, the *pagN* and *invA* genes strongly conserved but unique to *Salmonella* spp. were selected for developing the IMB-qPCR detection method in the present work.

There existed little cross-reactivity between anti-*PagN* polyclonal antibody prepared by rabbits and *Salmonella* isolates. The possible reason might have been due to the pre-existing low level *Salmonella* antibodies in rabbits caused by common *Salmonella* infection in humans and animals (Schadich *et al.*, 2016). However, the usage of anti-*PagN* polyclonal antibody-magnetic beads can greatly enhance the capture efficiency and improve sensitivity for detecting lower abundance *Salmonella* colonies. In addition, the high effect and affinity antibody would be obtained by immunising rabbits with specific proteins several times. Furthermore, the *Salmonella pagN*-deficient mutant strains could be used to absorb cross-reactivity antibodies from prepared polyclonal antibodies to achieve the preparation of highly specific and sensitive IMBs.

In the present work, IMBs were used to capture and enrich *Salmonella* (Chen and Park, 2018). We chose 1 - 2 μm carboxylated magnetic beads with high uniformity and dispersion to improve the capture ability and recovery efficiency of magnetic beads (Kato *et al.*, 2014). At the same time, the amount of IMBs plays a significant role in bacteria capturing. The real-time PCR coupled with either centrifugation (PCR-cen) or immunomagnetic separation (PCR-IMS) was used for the detection of *Salmonella* in fresh mung bean sprouts. The limit of detection was similar and enrichment of 10 h was sufficient for these two methods for *Salmonella* detection (Zheng *et al.*, 2016). In theory, the greater the amount of IMBs, the higher the capture efficiency. The optimal amount of IMBs is 0.2 mg, which not only achieve low cost but also maintain the *Salmonella* capture efficiency of 71.43 and 80.92% for pork and milk samples, respectively. The IMBs-qPCR method for artificial food samples detection showed a little reduction in the capture efficiency of *Salmonella*, which might have been due to the complexity of food matrix affecting the capture of IMBs for bacteria or the partial loss of magnetic beads. Therefore, we cultured the *Salmonella* from 10^1 to 10^4 - 10^5 CFU/mL to make up for the shortcomings of low capture efficiency indirectly in food samples. However, based on previous report, the capture efficiency of *Salmonella* was only about 50% using IMBs-qPCR method based on *pagC* gene in food samples (Wang *et al.*, 2018). In addition, for the artificial contamination of food, the selected culture conditions were conducive for bacterial growth which could promote bacterial proliferation and reduce the experiment time. As long as the concentration of *Salmonella* in the artificial simulation samples reaches 10^1 CFU/mL, the colonies would be detected by IMBs-qPCR method developed in the present work.

Conclusion

The traditional culture method to detect foodborne pathogens, typically requires enrichment culture, is complicated and time consuming. The main factors such as sensitivity, efficiency, and convenience determine whether a pathogen detection method will be useful or not in rapid detection for food safety incident or food factory (Zheng *et al.*, 2016). The IMBs-qPCR method developed herein could be used to detect the *Salmonella* when the colonies are no less than 10^1 CFU/mL within 9 h in food samples, which achieved the purpose of efficient, accurate, and quantitative detection. It has enriched the rapid detection techniques of foodborne pathogenic *Salmonella*, and laid the foundation for development and application of the bacterial detection kit.

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