

Development of IMBs-qPCR Detection Method for *Yersinia enterocolitica* Based on the *foxA* Gene

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Abstract

Yersinia enterocolitica is an important zoonotic pathogen, which seriously endangers food safety risk. In this study, the recombinant outer membrane protein OmpF and its antibody were prepared and coupled with immunomagnetic beads (IMBs) to capture *Y. enterocolitica* in food samples, combining the quantitative PCR detection with primers of virulence factor gene fox A for *Yersinia enterocolitica* contamination. The results showed that the capture efficiency of approximately 80% using anti-OmpF antibody-immunomagnetic beads and linearly dependent capture under 10^1 - 10^5 CFU/mL *Y. enterocolitica*. compare with less than 10% capture of other bacteria. The detection limit of 64 CFU/mL was obtained by based on fox A gene PCR detection combined with capture of the anti-OmpF antibody-immunomagnetic beads to detect *Yersinia enterocolitica* in artificially contaminated milk and pork samples. Comparing with the culture method, the developed IMBs-qPCR method has higher consistency, less time consuming, which providing an effective alternative method for rapid detection of *Y. enterocolitica* in food.

Introduction

Yersinia enterocolitica is a gram-negative bacteria with a size of 0.5 - $1.3 \mu\text{m} \times 1$ - $3 \mu\text{m}$, which can grow at 0 - 44°C , and the most suitable growth temperature is 20 - 28°C (Saraka et al. 2017). *Y. enterocolitica* is an important foodborne pathogen, which is mainly infected through the digestive tract, such as eating contaminated pork and dairy products(Fabrega and Vila 2012). After infection with *Y. enterocolitica*, the main symptoms of the patient are self-limiting gastroenteritis, lymphadenitis, and terminal ileum inflammation, but a few patients may also have serious complications such as sepsis and even died(Fredriksson-Ahomaa et al. 2006; Rosner et al. 2010). *Y. enterocolitica* has outbreaks all around the world(Shayegani et al. 1983; Morse et al. 1984), and it is currently listed as a routine testing item for imported and exported foods in many countries.

Rapid detection of pathogenic bacteria in food is essential to reduce food safety risks. The culture detection method is the most common method to detect *Y. enterocolitica*. However, the detection method has many steps, which take at least one week to obtain the report results(Thoerner et al. 2003). Moreover, the specificity and accuracy of the culture detection are not high, which cannot fully meet the actual detection needs of the food industry(Rusak et al. 2018). The outer membrane is a unique structure of the cell wall of Gram-negative bacteria, which has important biological functions for bacteria(Koebnik et al. 2000; Delcour 2009). Outer membrane protein (OMP) is a significant part of the outer membrane, and most of which are β -barrel structural proteins, composed of 8 to 24 β -sheets arranged in an anti-parallel pattern(Fairman et al. 2011). This special structure can maintain the good stability of OMPs and help bacteria to withstand complex and changeable external environments(Mikula et al. 2012). OMPs can not only exert their biological functions, but also have good immunogenicity, and are considered to be important candidate antigens for vaccines(Huang et al. 2016; Zhang et al. 2018). Immunomagnetic bead separation (IMS) technique is based on the specific reaction mechanism of antigen and antibody, which can achieve the specific capture of bacteria. In recent years, this technology is widely used to capture and

enrich bacteria in food samples, combined with ELISA, real-time quantitative PCR (qPCR) and other technologies to specifically detect bacteria (Zhu et al. 2011; Srisa-Art et al. 2018; Wang et al. 2018b). We couple antibodies against the OMP of *Yersinia* on the surface of immunomagnetic beads to specifically capture *Yersinia* in food samples.

The outer membrane protein attachment invasion locus (Ail) is only found in pathogenic *Yersinia* and is considered to be an important virulence factor (Pierson and Falkow 1993; Tsang et al. 2013). Ail protein can promote the ability of *Y. enterocolitica* to attach and invade cells and help to improve the resistance of bacteria to serum (Bliska and Falkow 1992). Outer membrane protein F (OmpF) plays an important role in controlling the selective penetration of cell membranes (Shaban et al. 2017) and is also considered as a candidate antigen for inhibiting *Y. enterocolitica* infection (Wang et al. 2018a). Both outer membrane proteins Ail and OmpF have strain-specificity and good conservation (Huang et al. 2010; Stenkova et al. 2011), therefore we chose their antibodies as coupling proteins of immunomagnetic beads.

Many studies have shown that the quantitative real-time PCR method can be applied to the detection of foodborne pathogenic bacteria with good specificity and repeatability (Kasturi and Drgon 2017; Vital et al. 2017; Wang et al. 2018b). The ferrioxamine receptor gene (*foxA*) is located on the chromosome of *Y. enterocolitica*, which combines with ferrioxamin to take up ferric ion (Perry and Brubaker 1979). The gene *foxA* is stable and conservative in *Y. enterocolitica* with high species specificity (Huang et al. 2010; Wang et al. 2014), therefore it is selected as the target gene of qPCR. In this research, we have established a detection method that combines immunomagnetic bead separation technology with qPCR technology, which can achieve rapid quantitative detection of bacteria in contaminated complex food matrices.

Materials And Methods

2.1 Bacterial strains, culture conditions and Animals

All *Yersinia enterocolitica* strains were cultured in modified phosphate buffer saline at 26°C, and other bacterial strains were cultured in Luria Broth (LB) broth at 37°C. All strains used in the experiment were stored in our laboratory and stored with glycerol (final concentration of 30%) in a refrigerator at -80°C. Two SPF adult rabbits were purchased from the Institute of Radiation Medicine of the Chinese Academy of Medical Sciences in Tianjin and handled in accordance with the guidelines for animal experiments of the University of Tianjin University and the Chinese Academy of Medical Sciences. **The research protocol was approved by the Animal Ethical and Welfare Committee of Tianjin University (Approval No. TJUE-2021-051).** The purified OmpF or Ail protein was injected into the rabbits by subcutaneous at a concentration of 1 mg/mL each time. A total of 4 immunizations were performed with a week interval. The Blood was collected 5 days after the last immunization and the sera were separated to prepared the purified antibody by the method of precipitation with saturated ammonium sulfate (pH7.0).

2.2 Expression and purification of recombinant outer membrane protein

The *ail* and *ompF* gene were amplified and sub-cloned into the vector pET28a to generate the fusion plasmid of pET28a-*ail* and pET28a-*ompF*. The primers were supplied in Table 2. The above sequences were verified by gene sequencing (Genewiz Corp, Beijing, China), and no mutations such as frameshift and gene deletion were detected. The expression and purification of recombinant Ail and OmpF by using the process described as our previous report(Kang HZ 2015). The protein samples were subjected to SDS-PAGE electrophoresis on 12% polyacrylamide gel using Mini-Protean (BioRad).

2.3 Preparation and purification of polyclonal antisera of recombinant outer membrane protein

The 5-month-old SPF rabbits were purchased from the Institute of Radiation Medicine Chinese Academy of Medical Sciences and were raised in specific pathogen-free facilities. We used 1 mg/mL purified recombinant outer membrane protein (Ail and OmpF) to generate polyclonal antisera. In the first immunization, 1 mL of protein with a concentration of 1 mg/mL was mixed with an equal volume of Freund's complete adjuvant, and subcutaneous immunization was performed at multiple locations on the back of the rabbits. Freund's incomplete adjuvant was used for 2-4 immunizations. The immunization procedure is the same as the first immunization, with an interval of 10 days. Rabbit blood was taken 10 days after the fourth immunization, and the antibody was purified by the saturated ammonium sulfate method. Subsequently, the antibody concentration and titer were determined through the Bradford method and indirect ELISA method. respectively.

2.4 Western-blot analysis of polyclonal antibodies

Two strains of *Y. enterocolitica* (CMCC 55075, CMCC52217) single colonies were picked and cultured in 5 mL of modified phosphate buffer at 26 °C and 150 rpm shaker overnight. 20 µL bacterial solution was made whole bacteria sample. In the prokaryotic expression and purification of OmpF and Ail proteins, whole bacteria samples were isolated by SDS-PAGE. Subsequently, the membrane was blocked with PBST and 5% horse serum (**Gibco, Lot 26050-088**) for 1 hour. The membrane was stained with primary antibodies (rabbit anti-OMP antiserum diluted at 1:800) overnight at 4°C, then it was washed with PBST and stained with secondary antibodies (HRP goat anti-rabbit IgG antibody diluted at 1:4000) for 1 hour. The membrane was detected by using a chemiluminescence detection kit (Thermo Scientific, Waltham, MA, USA) and observed through Gel Imaging System (BIO-RAD, USA).

2.5 Preparation of antibody-conjugated immunomagnetic beads

Carboxyl-modified Affimag PSC magnetic beads with mean diameter of 1.0 µm were purchased from Besile Technology (Tianjin, China). Unprocessed magnetic beads (2mg) were washed three times with 800 µL 0.01M phosphate buffer saline containing Tween 20 (PBST, pH 6.0). Then we resuspend the magnetic beads with 600 µL 0.01M PBST (pH 6.0), and slowly added 1-ethyl-3 carbodiimide (EDC) and N-Hydroxysuccinimide (NHS) to a final concentration of 5mg/mL. After fully shaking and mixing, the magnetic beads were activated in the shaker at room temperature for 30 min. After activation, the magnetic beads were washed three times with 800 µL 0.01M PBST (pH 7.4) and resuspended with 600 µL 0.01M PBST (pH 7.4). Purified antibodies were added slowly and placed in the shaker at room

temperature for 4 h to ensure the antibody and magnetic beads are fully bound. The antibody-bound magnetic beads were washed three times with 800 μ L 0.01M PBST (pH 7.4) and then blocked with 600 μ L 0.01M PBST (pH 7.4) with 1% BSA in a shaker at room temperature for 30 min. After washed three times with 800 μ L 0.01M PBST (pH 7.4), the antibody-conjugated immunomagnetic beads (IMBs) were resuspend with 500 μ L 0.01M PBST (pH 7.4) with 0.1% BSA and stored at 4°C in the refrigerator.

2.6 Optimization of preparation parameters of antibody-conjugated immunomagnetic beads

The dosage and coupling time of magnetic beads and antibodies were determined through preliminary experiments. Then we explored the capture efficiency of immunomagnetic beads coupled with OmpF or Ail antibodies of different qualities (0.005 mg, 0.01 mg, 0.02 mg, 0.05 mg, 0.1mg, 0.2 mg, 0.25 mg) to 1 mL of *Y. enterocolitica* with a concentration of 10^4 CFU/mL- 10^5 CFU/mL. And we compared and analyzed the difference between the capture efficiencies of the two antibody-conjugated immunomagnetic beads. After establishing the optimal antibody and conjugated immunomagnetic beads dosage, the capture efficiency of 1 mL series dilution concentration from 10^1 CFU/mL to 10^6 CFU/mL of *Y. enterocolitica* were further evaluated by PCR detection of the DNA extracted from the capturing bacteria..

2.7 Specific analysis of immunomagnetic bead separation

Y. enterocolitica (CMCC 55075), *Staphylococcus aureus* (ATCC 25923), *E. coli DH5 α* , *Salmonella typhimurium* (CMCC 50619) single colonies were picked and cultured in a culture medium overnight. The bacterial solution was gradient diluted with sterile PBS buffer, and the initial bacterial solution concentration was determined by colony counting method. Subsequently, we took 1 mL of bacteria with a concentration of 10^4 CFU/mL- 10^5 CFU/mL and added appropriate quality antibody-conjugated immunomagnetic beads to capture, and vortex for 40 minutes at room temperature. Place the immunomagnetic beads on a magnetic device (Besile Technology, Tianjin, China) and let stand for 3 minutes to aspirate the supernatant. After the supernatant was diluted with sterile PBS buffer, the concentration of the captured bacteria in the supernatant was measured by the colony counting method, and the capture efficiency of the antibody-conjugated immunomagnetic beads on different bacteria was calculated and compared.

2.8 Scanning electron microscope observation of IMBs-*Y. enterocolitica* complex

A single colony of *Y. enterocolitica* (CMCC 55075) was picked and cultured in 5 mL of modified phosphate buffer at 26 °C and 150 rpm shaker overnight and diluted to 10^4 CFU / mL with sterile PBS. They were captured with 0.2 mg of immunomagnetic beads conjugated with OmpF and Ail antibodies. 800 μ L of sterile water was added to wash the complex of antibody-conjugated immunomagnetic beads and bacteria twice and subsequently resuspended in 200 μ L of sterile water. 10 μ L of resuspended droplets was added onto the coverslip and placed in a 37 °C oven to remove water. After being completely air-dried, the samples were sprayed with gold and observed with a field emission scanning electron microscope (NOVA Nanosem 430, FEI, USA).

2.9 Quantification of *Y. enterocolitica* by qPCR

We use online software PrimerQuest (<https://www.idtdna.com/Primerquest/>), Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>), Beacon Free Edition (<http://www.premierbiosoft.com/>) to design the primers of the *foxA* gene (F: 5'-CATCCCTGGTGGTGTAGTA-3', R: 5'-GTTTCAGATATCGCATCGGTATAA-3', amplified length:131bp). This online software can ensure that the primers have good specificity and no obvious primer dimer formation. The entire reaction system was performed using a 20 μ L volume containing 10 μ L 2 \times TransStart Top Green qPCR SuperMix (TransGen Biotech, China), 0.4 μ L forward and reverse primers (10 μ M), 2 μ L DNA template, and 7.2 μ L distilled water. A three-step method was adopted in this experiment. The qPCR conditions for *foxA* gene were as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles at 54°C for 30 s to denaturation and 70°C for 20 s to renaturation. The qPCR assay was carried out using the Roche LightCycler®96 qPCR instrument (ROCHE GROUP, Switzerland). We diluted 10-fold the *Y. enterocolitica* (CMCC 55075) grown overnight with aseptic PBS buffer. Subsequently, we used bacterial genome extraction kit DNA (TianGen, China) to extract genomic DNA of 1 mL *Y. enterocolitica* at different dilutions, which was used as templates to establish the qPCR standard curve of *foxA* gene.

The specificity of this method was verified by interference experiments. *E. coli* DH5a, *Salmonella typhimurium* (CMCC 50619), and *Staphylococcus aureus* (ATCC 25923) were selected as interference strains. Dilute each strain after overnight cultivation, and then sequentially add it to *Y. enterocolitica* 10^2 - 10^6 CFU / mL in sequence. 1 mL of the mixed solution was taken to extract genomic DNA (100 μ L). It is used as a template to perform qPCR detection according to the above reaction procedure and system and analyze the specificity of the detection method.

2.10 IMBs-qPCR detects *Y. enterocolitica* in food

Whole milk and pork were purchased from the local market, which was used to artificially simulate food contaminated by *Y. enterocolitica*. We used UV sterilization to ensure that bacteria in milk and pork were eliminated. 25 g test sample (milk/pork) was taken into an flask containing 250 mL of modified phosphate buffer, after fully shaking and mixing, added *Y. enterocolitica* diluted with sterile PBS to the final concentration of about 10 CFU / mL, placed in a shaker at 26 °C for 5h to enrich bacteria. 0.2 mg of antibody-conjugated immunomagnetic beads were mixed in 1mL enriched food samples, and the mixture was shaken at room temperature for 40 min. After fully absorbing the antibody-conjugated immunomagnetic beads with a magnetic device, bacterial genomic DNA (100 μ L) was extracted, and qPCR detection was performed according to the reaction procedure and system of 2.9. The enriched sample and supernatant after immunomagnetic bead capture were multiple diluted and colony counted. The results of the IMBs-qPCR method were compared with the results of the colony counting method, and the accuracy of the fluorescent quantitative PCR method was evaluated.

2.11 Statistical analysis

At least three independent repeated experiments were carried out for the above experiments. Statistical significance among different results was determined by GraphPad Prism software using t-test. P values less than 0.05 were considered statistically significant. (*p < 0.05; **p < 0.01; ***p < 0.001).

Results

3.1 Prokaryotic expression of recombinant outer membrane proteins have high concentration and purity

The distribution of *ail* and *ompF* genes in 13 *Yersinia* strains was evaluated by PCR analysis using specific primers. The results showed that the *ail* gene can be amplified only in *Y. enterocolitica*, while the *ompF* gene can be amplified in all 13 *Yersinia* strains (Table 1). The recombinant proteins of Ail and OmpF purified by nickel column affinity chromatography were analyzed by SDS-PAGE. The results show that the size of purified protein was consistent with the predicted results. What is more, there are no obvious mixed proteins (Figure 1). The concentrations of Ail protein and OmpF protein were 5 mg/mL, which can meet the needs of subsequent immunization experiments.

3.2 Polyclonal antibody and titer determination and Western blot analysis

The indirect ELISA method was used to determine the titers of purified Ail and OmpF antibody, and the P/N ratio method was used to determine the ELISA results. The calculation formula is: P/N = test serum OD value / negative serum OD value. In this formula, when P/N ≥ 2.1, it can be determined that the test serum is positive. The purified indirect ELISA results of Ail and OmpF antibodies are shown in Figure 2. The antibody titers of Ail and OmpF are 1: 3200, which had relatively high titers.

Western blot analysis further verified the specificity of Ail and OmpF polyclonal antibodies and the results are shown in Figure 2. The results showed that the two antibodies prepared by the experiment can not only bind to the recombinant protein expressed in prokaryotic cells but also specifically bind to the natural outer membrane proteins Ail and OmpF in *Y. enterocolitica* to meet the needs of subsequent experiments.

3.3 Capture efficiency analysis of IMBs

Through preliminary experiments, we determined that the amount of antibody that can be coupled to 1 mg of immunomagnetic beads is 112 µg, and the optimal time for coupling antibodies to immunomagnetic beads is 6 h. The capture efficiency of different qualities of IMBs (0.005 mg, 0.01 mg, 0.02 mg, 0.05 mg, 0.1 mg, 0.2 mg, 0.25 mg) coupled with OmpF and Ail antibodies against *Y. enterocolitica* (1.58×10^4 CFU/mL) is shown in Figure 3. The platform period is basically reached when the amount of IMBs used is 0.2 mg. The capture efficiency of IMBs coupled with OmpF antibody is basically 80% (Figure 3A), and the capture ability of IMBs coupled with Ail antibody is close to 70% (Figure 3B). The difference in the capture capacity of the two antibodies may be related to the size of the antigen protein and the number of epitopes. The capture efficiency of the magnetic beads was not significantly improved when the amount of IMBs increased from 0.2 mg to 0.25 mg. Considering the

economic benefits, 0.2 mg of IMBs was selected as the prescribed amount for capturing per milliliter samples.

0.2 mg IMBs coupled with OmpF antibody were used to capture 1 mL of *Y. enterocolitica* with different concentrations (6.31×10^1 CFU/mL - 6.31×10^6 CFU/mL). The capture efficiency is shown in Figure 4: $69.12 \pm 4.32\%$, $72.34 \pm 3.33\%$, $75.12 \pm 3.42\%$, $80.98 \pm 3.10\%$, $63.66 \pm 5.11\%$, $33.56 \pm 4.10\%$, respectively. When the bacterial concentration was 6.31×10^1 CFU/mL - 6.31×10^5 CFU/mL, the capture efficiency was relatively stable, close to 70%. The capture efficiency was the highest at 6.31×10^4 CFU/mL, reaching 80%.

3.4 Specific analysis and scanning electron microscope observation of IMBs

The capture efficiency of 0.2 mg IMBs for 10^4 - 10^5 CFU/mL other different bacteria was shown in Figure 4. The capture efficiency of IMBs for *Y. enterocolitica*, *E. coli*, *Staphylococcus aureus*, and *Salmonella typhimurium* is $76.45 \pm 1.5\%$, $9.11 \pm 0.79\%$, $7.12 \pm 1.06\%$, $4.90 \pm 0.98\%$, respectively. The results showed that the IMBs prepared in this experiment capture *Y. enterocolitica* significantly more efficiently than other strains and have good species specificity.

The scanning electron microscope observation of *Y. enterocolitica* captured by IMBs is shown in Figure 5. Figure 5A shows *Y. enterocolitica* CMCC 55075 and Figure 5B shows IMBs after antibody coupling. It can be observed that the surface of the IMBs is uneven due to antibody coating; Figure 5C and 5D respectively display the complexes of OmpF and Ail antibody-conjugated magnetic beads to capture *Y. enterocolitica* CMCC 55075. Through scanning electron microscope (SEM) observation, it can be concluded that the IMBs prepared in this experiment can effectively capture *Y. enterocolitica*.

3.5 Determining the standard curve of qPCR and detection specificity

The Ct value of each template in qPCR is linearly related to the logarithm of the initial copy number of the template (Galluzzi et al. 2018), $Ct = -k \log X_0 + b$ (X_0 is the initial copy number of the template). The standard curve of *foxA* gene was established by *Y. enterocolitica* (CMCC 55075) with a concentration of 6.4×10^1 - 6.4×10^7 CFU/mL. The standard curve is $y = -3.4601x + 41.01$, the amplification efficiency is 95 percent, $R^2 = 0.997$, and the detection limit is 64 CFU/mL.

The specificity of the detection method was verified by interference experiments, and *E. coli DH5a*, *Salmonella typhimurium* (CMCC 50619), and *Staphylococcus aureus* (ATCC 25923) were selected as interference strains. The results (Figure 6) showed that the amplification curve was not significantly affected after the interfering strain was added, indicating that the background strains did not affect the amplification of the *foxA* gene of *Y. enterocolitica*, which proved that the *foxA* gene detection primer designed in this experiment had good specificity.

3.6 Detection and quantitative *Y. enterocolitica* in food samples by IMBs-qPCR

Whole milk and pork are inoculated *Y. enterocolitica* to simulate contaminated food samples. The food inoculated with about 10 CFU/mL enterococci was enriched in a modified phosphate buffer at 26°C for 6 hours, then the anti-OmpF antibodies-conjugated immunomagnetic beads were captured and the bacterial genome was extracted to complete the qPCR detection. The results of the IMBs-qPCR method were compared with the results of the colony counting method.

The capture efficiency of *Y. enterocolitica* in whole milk and pork samples through the IMBs-qPCR method was 78.71% and 72.45% (Table 3). It may be that the tissues present in the pork occupy part of the epitope of the immunomagnetic beads, resulting in reduced capture efficiency(Xiong and Cui 2014).

Discussion

The immunomagnetic separation (IMS) technology has been widely used in the detection of pathogenic bacteria in food(Lim et al. 2016; Chen and Park 2018; Song et al. 2018). Outer membrane protein (OMP) is a significant component of Gram-negative bacteria, which has important biological functions and immunogenicity(Wen et al. 2016; Chen et al. 2017; Schrammel et al. 2018). In our research, based on the detection of virulence genes of several strains of *Y. enterocolitica* and the bioinformatics analysis of Ail and OmpF bioinformatics of specific outer membrane protein genes of Enterococcus. The recombinant outer membrane proteins Ail and OmpF at a concentration of 5 mg/mL were purified, and polyclonal antibodies were prepared by immunizing rabbits. ELISA and Western blot assays revealed that the polyclonal antibodies prepared from recombinant outer membrane protein have high titer and specificity.

The parameters of immunomagnetic beads preparation were optimized through experiments, and the optimal time for magnetic beads coupling was determined to be 6 h. For every 1 mg of immunomagnetic beads coupled, 200 µg of antibody was added. 0.2 mg IMBs coupled with OmpF antibody were used to capture 1 mL of *Y. enterocolitica* with different concentrations samples owing to that the platform period is basically reached when the amount of IMBs used is 0.2 mg. The immunomagnetic beads prepared in this experiment have good specificity. When the bacterial concentration is 10^4 - 10^5 CFU/mL, the capture efficiency of IMBs for *Y. enterocolitica* is higher than 80%, at the time, the capture efficiency for other strains is less than 10%. In addition, we found that the maximum capture efficiency (80%) of the IMBs prepared with OmpF polyclonal antibody was higher than the IMBs prepared with Ail polyclonal antibody (70%). The difference in capture efficiency between the two polyclonal antibodies may be mainly due to the molecular weight difference between OmpF (42 kDa) and Ail (20 kDa), and more antigenic epitopes existed in OmpF protein than that of Ail protein. The increase of antigen epitopes can improve the contact site of immunomagnetic beads and bacteria surface, and promote the binding of bacteria and antibody. Moreover, the expression of Ail protein in *Y. enterocolitica* is greatly impacted by the ambient temperature, and the protein expression level at room temperature is low, which is not conducive to the capture of IMBs(Bliska and Falkow 1992).

The real-time quantitative PCR (qPCR) for specific detection of bacteria has received widespread attention(Garrido-Maestu et al. 2018; Agrimonti et al. 2019). The ferrioxamine receptor gene (*foxA*) is

relatively conserved in *Y. enterocolitica* and has a high bacterial species specificity (Kornreich-Leshem et al. 2005; Huang et al. 2010). To improve the sensitivity of IBM-qPCR therefore, it was selected as the fluorescent quantitative PCR detection gene. Based on online software, the qPCR primers for *foxA* gene with good specificity and non-specific amplification were designed with a detection limit is 64 CFU/mL.

The IMBs-qPCR method was used to detect *Y. enterocolitica* in artificially contaminated milk and pork, and the capture rate was greater than 70% when the bacterial concentration was 10^3 - 10^4 CFU/mL. Compared with the cultivation method, the IMBs-qPCR method has higher consistency and the whole process only takes 12 hours, which greatly shortens the detection time. In summary, we had established a detection method based on the IMBs-qPCR method, which can rapidly and accurately detect *Y. enterocolitica* in food and can be applied to emergency food safety incidents.

Declarations

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Author Contributions

Conceived and designed the experiments: JH H. Performed the experiments: M Z, H C, AP C, LL Z and FZ X. Analyzed the data: AP C and JH H. Contributed reagents/materials /analysis tools: JH H. Wrote the paper: H C, and JH H.

Author Conflict of Interest

We declare that we have no competing interests.

References

Agrimonti C, Bottari B, Sardaro MLS, Marmiroli N (2019) Application of real-time PCR (qPCR) for characterization of microbial populations and type of milk in dairy food products. *Crit Rev Food Sci Nutr* 59:423-442 doi: 10.1080/10408398.2017.1375893

Bliska JB, Falkow S (1992) Bacterial resistance to complement killing mediated by the Ail protein of *Yersinia enterocolitica*. *Proc Natl Acad Sci U S A* 89:3561-3565 doi: 10.1073/pnas.89.8.3561

Chen J, Park B (2018) Effect of immunomagnetic bead size on recovery of foodborne pathogenic bacteria. *Int J Food Microbiol* 267:1-8 doi: 10.1016/j.ijfoodmicro.2017.11.022

- Chen Q, Li Y, Tao T, Bie X, Lu F, Lu Z (2017) Development and application of a sensitive, rapid, and reliable immunomagnetic separation-PCR detection method for *Cronobacter* spp. *J Dairy Sci* 100:961-969 doi: 10.3168/jds.2016-11087
- Delcour AH (2009) Outer membrane permeability and antibiotic resistance. *Biochim Biophys Acta* 1794:808-816 doi: 10.1016/j.bbapap.2008.11.005
- Fabrega A, Vila J (2012) *Yersinia enterocolitica*: pathogenesis, virulence and antimicrobial resistance. *Enferm Infecc Microbiol Clin* 30:24-32 doi: 10.1016/j.eimc.2011.07.017
- Fairman JW, Noinaj N, Buchanan SK (2011) The structural biology of beta-barrel membrane proteins: a summary of recent reports. *Curr Opin Struct Biol* 21:523-531 doi: 10.1016/j.sbi.2011.05.005
- Fredriksson-Ahomaa M, Stolle A, Korkeala H (2006) Molecular epidemiology of *Yersinia enterocolitica* infections. *FEMS Immunol Med Microbiol* 47:315-329 doi: 10.1111/j.1574-695X.2006.00095.x
- Galluzzi L, Ceccarelli M, Diotallevi A, Menotta M, Magnani M (2018) Real-time PCR applications for diagnosis of leishmaniasis. *Parasit Vectors* 11:273 doi: 10.1186/s13071-018-2859-8
- Garrido-Maestu A, Azinheiro S, Carvalho J, Prado M (2018) Rapid and sensitive detection of viable *Listeria monocytogenes* in food products by a filtration-based protocol and qPCR. *Food Microbiol* 73:254-263 doi: 10.1016/j.fm.2018.02.004
- Huang W et al. (2016) Immunization with a 22-kDa outer membrane protein elicits protective immunity to multidrug-resistant *Acinetobacter baumannii*. *Sci Rep* 6:20724 doi: 10.1038/srep20724
- Huang Y et al. (2010) Possible use of *ail* and *foxA* polymorphisms for detecting pathogenic *Yersinia enterocolitica*. *BMC Microbiol* 10:211 doi: 10.1186/1471-2180-10-211
- Kang HZ, DH, Shen ML, et al. (2015) Superantigenicity analysis of staphylococcal enterotoxins SEIK and SEIQ in a mouse model. *Rsc Advances* 5:29684-29692
- Kasturi KN, Drgon T (2017) Real-Time PCR Method for Detection of *Salmonella* spp. in Environmental Samples. *Appl Environ Microbiol* 83 doi: 10.1128/aem.00644-17
- Koebnik R, Locher KP, Van Gelder P (2000) Structure and function of bacterial outer membrane proteins: barrels in a nutshell. *Mol Microbiol* 37:239-253 doi: 10.1046/j.1365-2958.2000.01983.x
- Kornreich-Leshem H et al. (2005) Ferrioxamine B analogues: targeting the *FoxA* uptake system in the pathogenic *Yersinia enterocolitica*. *J Am Chem Soc* 127:1137-1145 doi: 10.1021/ja035182m
- Lim MC et al. (2016) Biological preparation of highly effective immunomagnetic beads for the separation, concentration, and detection of pathogenic bacteria in milk. *Colloids Surf B Biointerfaces* 145:854-861 doi: 10.1016/j.colsurfb.2016.05.077

- Mikula KM, Kolodziejczyk R, Goldman A (2012) Yersinia infection tools-characterization of structure and function of adhesins. *Front Cell Infect Microbiol* 2:169 doi: 10.3389/fcimb.2012.00169
- Morse DL, Shayegani M, Gallo RJ (1984) Epidemiologic investigation of a Yersinia camp outbreak linked to a food handler. *Am J Public Health* 74:589-592 doi: 10.2105/ajph.74.6.589
- Perry RD, Brubaker RR (1979) Accumulation of iron by yersiniae. *J Bacteriol* 137:1290-1298
- Pierson DE, Falkow S (1993) The ail gene of Yersinia enterocolitica has a role in the ability of the organism to survive serum killing. *Infect Immun* 61:1846-1852
- Rosner BM, Stark K, Werber D (2010) Epidemiology of reported Yersinia enterocolitica infections in Germany, 2001-2008. *BMC Public Health* 10:337 doi: 10.1186/1471-2458-10-337
- Rusak LA et al. (2018) Rapid detection of Yersinia enterocolitica serotype O:3 using a duplex PCR assay. *J Microbiol Methods* 154:107-111 doi: 10.1016/j.mimet.2018.10.014
- Saraka D et al. (2017) Yersinia enterocolitica, a Neglected Cause of Human Enteric Infections in Cote d'Ivoire. *PLoS Negl Trop Dis* 11:e0005216 doi: 10.1371/journal.pntd.0005216
- Schrammel B, Petzold M, Cervero-Arago S, Sommer R, Luck C, Kirschner A (2018) Persistent presence of outer membrane epitopes during short- and long-term starvation of five Legionella pneumophila strains. *BMC Microbiol* 18:75 doi: 10.1186/s12866-018-1220-x
- Shaban H, Na I, Kislichkina AA, Dentovskaya SV, Anisimov AP, Uversky VN (2017) Effect of natural polymorphism on structure and function of the Yersinia pestis outer membrane porin F (OmpF protein): a computational study. *J Biomol Struct Dyn* 35:2588-2603 doi: 10.1080/07391102.2016.1224734
- Shayegani M, Morse D, DeForge I, Root T, Parsons LM, Maupin PS (1983) Microbiology of a major foodborne outbreak of gastroenteritis caused by Yersinia enterocolitica serogroup O:8. *J Clin Microbiol* 17:35-40
- Song X, Shukla S, Kim M (2018) Detection of Cronobacter species in powdered infant formula using immunoliposome-based immunomagnetic concentration and separation assay. *Food Microbiol* 72:23-30 doi: 10.1016/j.fm.2017.11.002
- Srisa-Art M, Boehle KE, Geiss BJ, Henry CS (2018) Highly Sensitive Detection of Salmonella typhimurium Using a Colorimetric Paper-Based Analytical Device Coupled with Immunomagnetic Separation. *Anal Chem* 90:1035-1043 doi: 10.1021/acs.analchem.7b04628
- Stenkova AM, Isaeva MP, Shubin FN, Rasskazov VA, Rakin AV (2011) Trends of the major porin gene (ompF) evolution: insight from the genus Yersinia. *PLoS One* 6:e20546 doi: 10.1371/journal.pone.0020546

- Thoerner P et al. (2003) PCR detection of virulence genes in *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* and investigation of virulence gene distribution. *Appl Environ Microbiol* 69:1810-1816 doi: 10.1128/aem.69.3.1810-1816.2003
- Tsang TM, Wiese JS, Felek S, Kronshage M, Krukoni ES (2013) Ail proteins of *Yersinia pestis* and *Y. pseudotuberculosis* have different cell binding and invasion activities. *PLoS One* 8:e83621 doi: 10.1371/journal.pone.0083621
- Vital PG, Van Ha NT, Tuyet LT, Widmer KW (2017) Application of quantitative real-time PCR compared to filtration methods for the enumeration of *Escherichia coli* in surface waters within Vietnam. *J Water Health* 15:155-162 doi: 10.2166/wh.2016.173
- Wang E et al. (2018a) Molecular Characterization, Phylogenetic, Expression, and Protective Immunity Analysis of OmpF, a Promising Candidate Immunogen Against *Yersinia ruckeri* Infection in Channel Catfish. *Front Immunol* 9:2003 doi: 10.3389/fimmu.2018.02003
- Wang J et al. (2018b) Rapid detection of food-borne *Salmonella* contamination using IMBs-qPCR method based on pagC gene. *Braz J Microbiol* 49:320-328 doi: 10.1016/j.bjm.2017.09.001
- Wang JZ et al. (2014) Real-time TaqMan PCR for *Yersinia enterocolitica* detection based on the ail and foxA genes. *J Clin Microbiol* 52:4443-4444 doi: 10.1128/jcm.02528-14
- Wen Z et al. (2016) Recombinant expression of *Chlamydia trachomatis* major outer membrane protein in *E. Coli* outer membrane as a substrate for vaccine research. *BMC Microbiol* 16:165 doi: 10.1186/s12866-016-0787-3
- Xiong Q, Cui X (2014) Development of an immunomagnetic separation method for efficient enrichment of *Escherichia coli* O157:H7. *Food Control* 37:41-45
- Zhang X et al. (2018) Immunization with *Pseudomonas aeruginosa* outer membrane vesicles stimulates protective immunity in mice. *Vaccine* 36:1047-1054 doi: 10.1016/j.vaccine.2018.01.034
- Zhu P et al. (2011) Detection of *E. coli* O157:H7 by immunomagnetic separation coupled with fluorescence immunoassay. *Biosens Bioelectron* 30:337-341 doi: 10.1016/j.bios.2011.09.029

Tables

Table 1 Primers used for prokaryotic expression vector construction

Primer name	Sequence 5'-3'	Length/bp	Tm/°C
pET-28a- <i>ail</i> -F	CAGCAAATGGGTCGCGGATCCGCGAGTGAAAATAGTATTTCTATTGGTT	468	58
pET-28a- <i>ail</i> -R	ACGGAGCTCGAATTCGGATCCTTAGAATAGATACCCTGCACCAAGTATC		
pET-28a- <i>ompF</i> -F	CAGCAAATGGGTCGCGGATCCGCGAGAAATCTACAACAAAGACGGC ACGGAGCTCGAATTCGGATCCTTAGAACTGGTAAACCAAGCCAAC	1050	58
pET-28a- <i>ompF</i> -R			

Table 2 The distribution of *ail* and *ompF* genes in 13 *Yersinia* strains

Bacterial strains	Source (origin)	<i>ompF</i>	<i>ail</i>
<i>Yersinia enterocolitica</i>	CMCC 50301	+	+
<i>Yersinia enterocolitica</i>	CMCC 52205	+	+
<i>Yersinia enterocolitica</i>	ATCC 49397	+	+
<i>Yersinia enterocolitica</i>	CMCC 52217	+	+
<i>Yersinia enterocolitica</i>	CMCC 55075	+	+
<i>Yersinia enterocolitica</i>	CMCC 52230	+	+
<i>Yersinia enterocolitica</i>	CMCC 52220	+	-
<i>Yersinia aldovae</i> Bercovier	ATCC 35236	+	-
<i>Yersinia kristensenii</i> Bercovier	ATCC 33637	+	-
<i>Yersinia frederiksenii</i>	ATCC 29912	+	-
<i>Yersinia mollaretii</i>	ATCC 43969	+	-
<i>Yersinia pseudotuberculosis</i>	ATCC 14143	+	-
<i>Yersinia rohdei</i>	ATCC 43871	+	-

Table 3 Capture efficiency of *Y. enterocolitica* in whole milk and pork samples by IMBs

Samples	α (CFU/mL)	β (CFU/mL)	γ (CFU/mL)	CE ₁ (%)	CE ₂ (%)	R
Pork	1.43×10 ⁴	3.94×10 ³	1.01×10 ⁴	72.45	70.63	0.97
Milk	9.11×10 ³	1.94×10 ³	7.55×10 ³	78.71	82.87	1.05

Note: α : The total number of *Y. enterocolitica* in 1mL of food sample after 6h of enrichment in modified phosphate buffer.

β : The total number of *Y. enterocolitica* in 1mL of supernatant after 40 minutes of capture at room temperature.

γ : The number of *Y. enterocolitica* detected by qPCR.

CE (%): The capture efficiency of immunomagnetic beads. CE_1 (%) = $(a-b) / a \times 100\%$; CE_2 (%) = $c / a \times 100\%$

R: Correlation coefficient. $R = CE_2$ (%) / CE_1 (%)

Figures

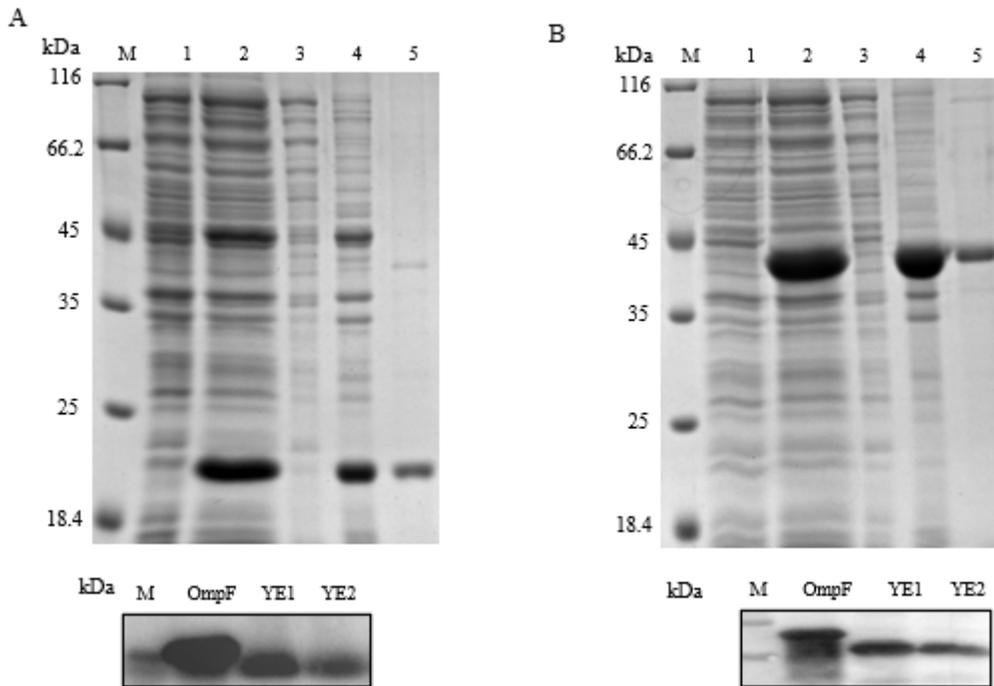


Figure 1

SDS-PAGE (upper) and Western-blot (lower) analysis expression and purification of recombinant Ail (A) or OmpF (B) protein. Lane M, protein Marker (thermo scientific, USA); lane 1 and lane 2, total cellular protein before and after IPTG induction; lane 3 and lane 4, the supernatant and precipitation of ultrasound pyrolysis of the sample after IPTG induction, respectively; lane 5, the purified Ail or OmpF protein; YE1, YE2 represent extract protein from *Y. enterocolitica* CMCC 55075 and CMCC 52217, respectively.

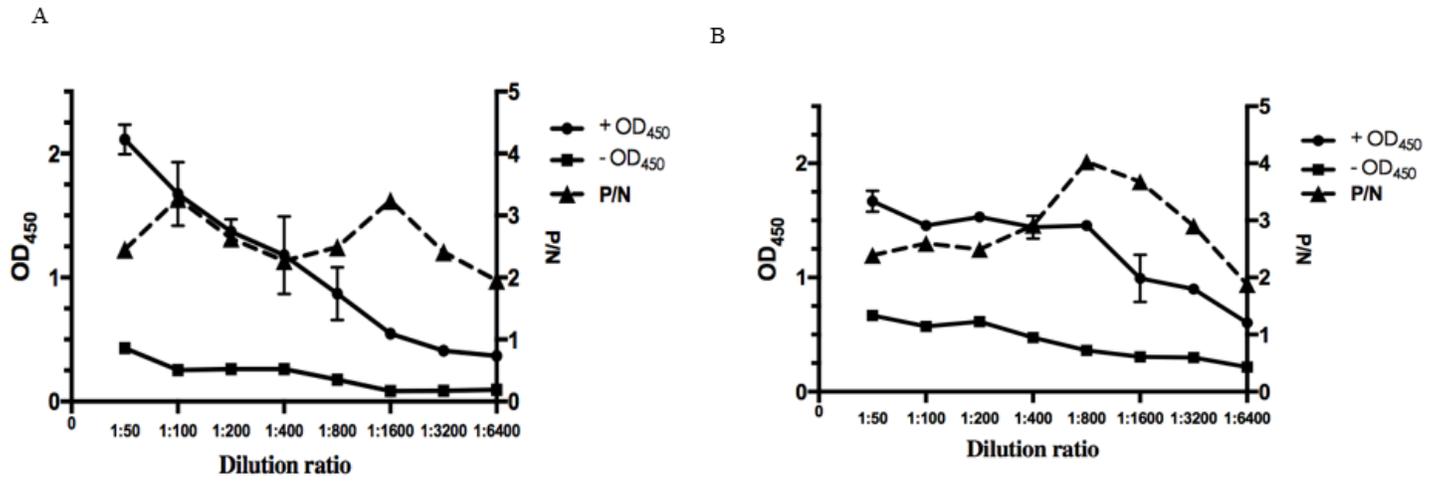


Figure 2

The ELISA titer detection results of rabbit anti-Ail (A) or OmpF (B) antibody.

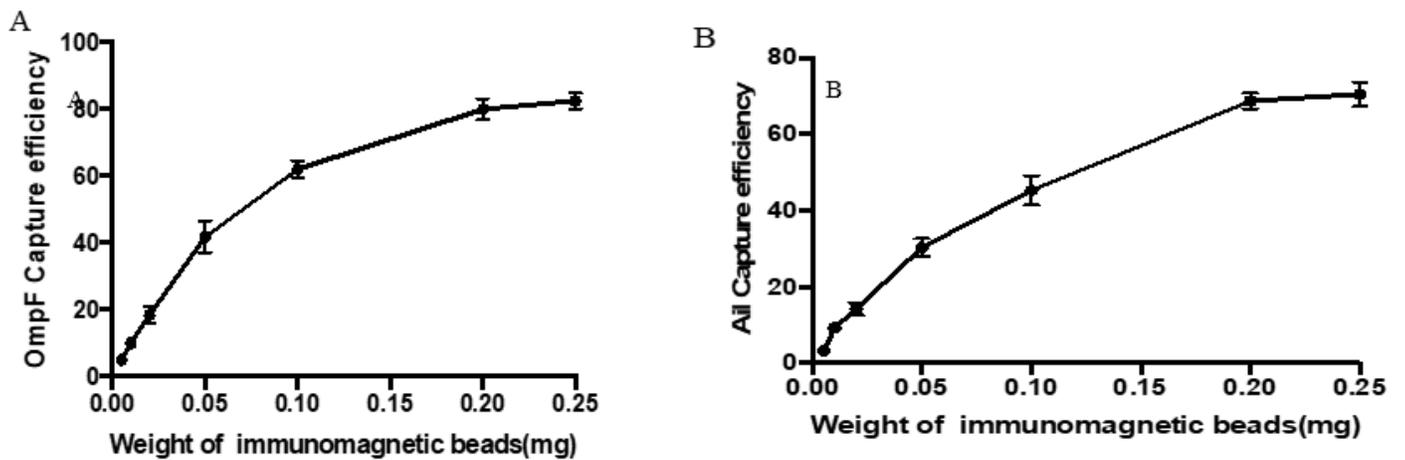


Figure 3

The capture efficiency of immunomagnetic beads coupling with rabbit anti-Ail (A) or OmpF (B) antibody for *Y. enterocolitica*.

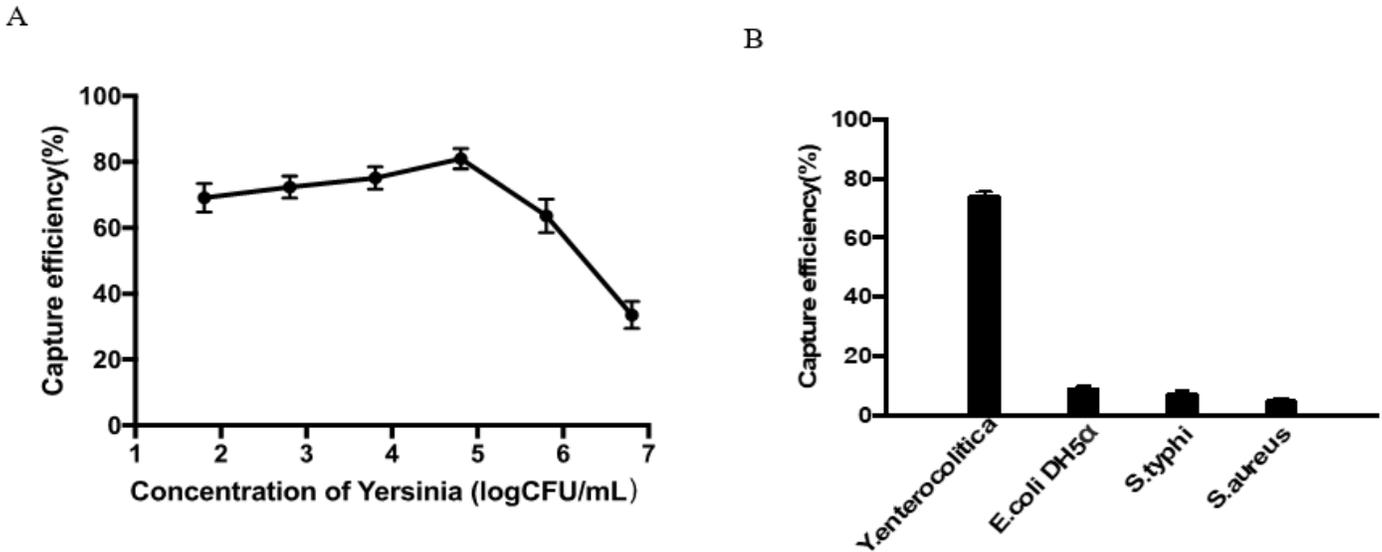


Figure 4

The capture efficiency change of anti-OmpF antibody-coupling immunomagnetic beads with different *Y. enterocolitica* concentration (A) and recognition of *Y. enterocolitica* and other bacterial species (B).

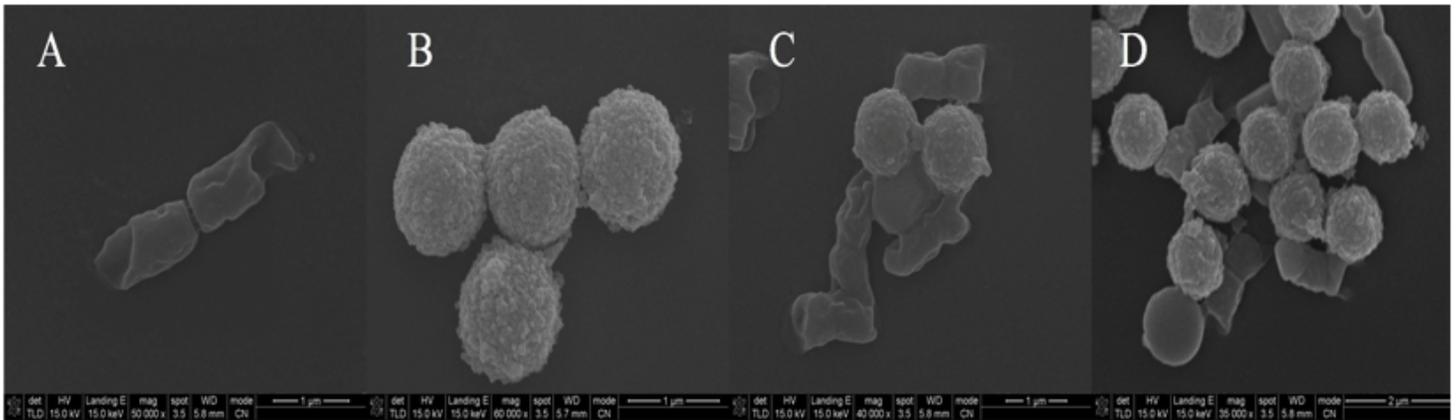


Figure 5

The scanning electron microscope photographs of anti-OmpF antibody-coupling immunomagnetic beads with *Y. enterocolitica* CMCC 55075. (A) *Y. enterocolitica* CMCC 55075; (B) anti-OmpF antibody-coupling immunomagnetic beads; (C) *Y. enterocolitica* CMCC 55075 was captured by anti-Ail antibody-coupling immunomagnetic beads; (D) *Y. enterocolitica* CMCC 55075 was captured by anti-OmpF antibody-coupling immunomagnetic beads.

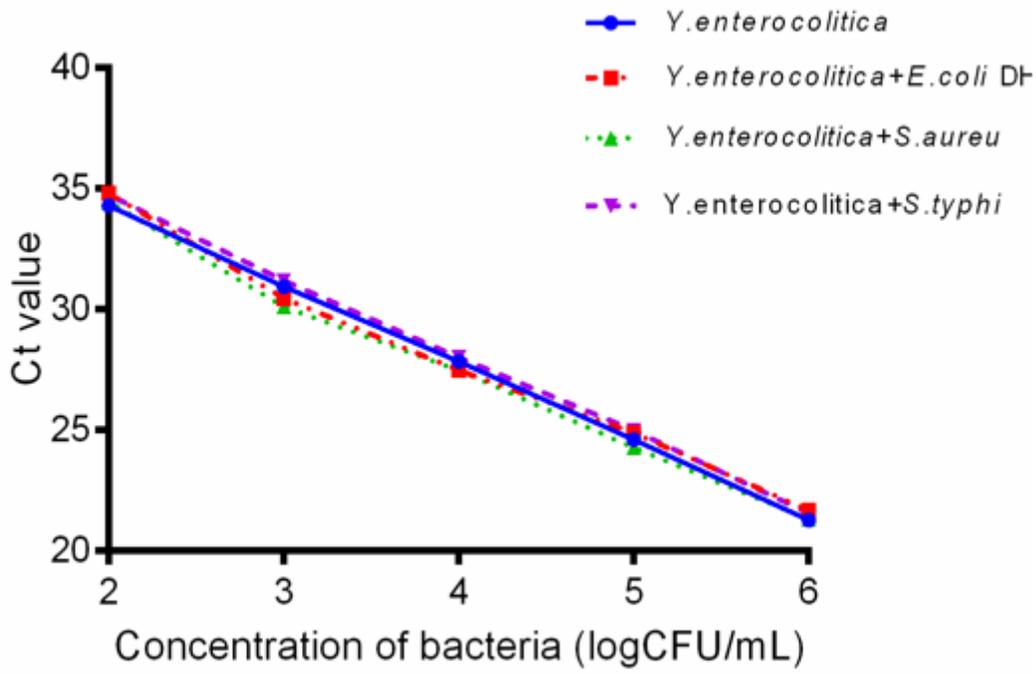


Figure 6

The IBM-qPCR detection results for *foxA* gene of *Y. enterocolitica* under artificial mixing *Escherichia coli*, *S. aureus*, or *Salmonella*.