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# A novel magnetic capture–multiplex PCR assay for the simultaneous detection of three foodborne pathogens

Haojiang Zuo[1], Zhimei Xie[1], Xiaobei Ding[1], Weiwei Zhang[1], Jingyan Yang[1], Xuejun Fan[2], Roland Poms[3,4] & Xiaofang Pei[1]

1 Department of Medical Technology, West China School of Public Health, Sichuan University, Chengdu, Sichuan, China
2 Sichuan Entry-Exit Inspection and Quarantine Bureau, Chengdu, Sichuan, China
3 ICC – International Association for Cereal Science and Technology, Vienna, Austria
4 ESR – Institute of Environmental Science and Research, Christchurch, New Zealand

**Abstract**

*Introduction Salmonella*, *Shigella* and *Escherichia coli* O157: H7 are major foodborne pathogens that cause gastrointestinal diseases worldwide. Apart from food contamination, fecal pollution has been consistently associated with the transmission of these pathogens, and their rapid detection in food and stools is of significance for food safety. However, a variety of factors associated with these complex samples can decrease the sensitivity and specificity of molecular-based methods for detection of these pathogens. *Objectives* The aim of this study was to develop a DNA-based method for the simultaneous detection of *E. coli* O157: H7, *Salmonella* and *Shigella* in stool and food samples. *Methods* In this study, a novel magnetic capture–multiplex polymerase chain reaction (PCR) assay was developed and its potential to detect the target pathogens in stool and food samples (including chicken, cucumbers and cooked rice) was tested. *Results* The results showed that the magnetic particles (MPs) used in the study had a high capacity for bacterial adsorption. The pretreatment protocol, which included the pathogen concentration by MPs, was developed and the sensitivity of the assay was approximately $10^0$ colony-forming unit (CFU) $g^{-1}$ in food and 1–10 CFUs per stool sample, following an enrichment step. The assay could be completed within 12 h, and was comparable in performance with conventional culture methods, which require several days to complete. *Conclusion* The assay combines MP-based magnetic capture with multiplex PCR, and offers an efficient, rapid, sensitive and inexpensive alternative for the routine detection of foodborne pathogenic bacteria.

## Introduction

*Salmonella*, *Shigella* and *Escherichia coli* O157: H7 are the most prevalent pathogenic bacteria in developing and developed countries. These pathogens account for a large number of the incidents of foodborne bacterial diseases (Li & Mustapha, 2004; Baldauf *et al.*, 2007; Sofos, 2008;

Mahmoud, 2009). Because fecal contamination has been consistently associated with the transmission of these pathogens (Niyog, 2005; Alakomi & Saarela, 2009; Hedican *et al.*, 2009; Saeed & Hamid, 2010), investigation of these pathogens in stool and food samples is very important to rapidly identify the source of contamination and to establish appropriate measures in order to reduce their

prevalence and risk. To improve food safety, the Food Safety Law of People's Republic of China requires service staff involved in the production of food to have annual health checks and examination of their feces for common foodborne pathogens, which are important indicators (http://www.gov.cn/flfg/2009-02/28/content_1246367.htm). The standard methods applied to pathogen detection remain conventional culture assays, which are time consuming and labor intensive.

New methods for pathogen detection, including polymerase chain reaction (PCR)-based approaches, aim to establish sensitive, high-speed and simultaneous detection systems (Li & Mustapha, 2004; Fiore *et al.*, 2010). However, when applied to the detection of pathogens in complex samples, such as feces or foods, the sensitivity and specificity of PCR-based assays can be interfered by sample components. A solution to this problem is to concentrate and separate the pathogens from the samples. To this end, magnetism-based capture techniques, including the use of magnetic beads linked with polyclonal or specific monoclonal antibodies (immunomagnetic beads), have been applied to pathogen concentration and separation (Xiao *et al.*, 2007; Cheng *et al.*, 2009; Yang *et al.*, 2010). However, unwanted cross reactions, poor reproducibility of polyclonal antibodies and strict specificity of monoclonal antibodies have limited their application to the simultaneous separation of different pathogenic bacteria. To overcome these problems, a magnetic carrier immobilized with a mixture of various monoclonal and polyclonal antibodies has enabled the capture of a wide range of bacterial serovars (Safařík *et al.*, 1995). Nevertheless, the complicated preparation procedure and relatively high cost involved have limited the application of this technique in large-scale surveillance and routine detection. Although not widely reported, magnetic particles (MPs) without polyclonal or specific monoclonal modification have the potential for high capture efficiency, short separation time and low-cost application to bacterial adsorption and concentration (Yang *et al.*, 2009). However, the combination of unmodified MPs with PCR-based methods in pathogen detection has not been reported.

In this study, unmodified MPs were prepared in our lab. These particles showed high capture efficiency for bacterial cells and demonstrated their potential and versatility for the simultaneous capture of different pathogens. Because multiplex PCR allows rapid, specific and inexpensive detection of several pathogens in a single reaction, and has widely been applied in the detection of foodborne pathogens, the combination of MPs with multiplex PCR represents a promising

**Table 1** Target and non-target bacterial strains used for specificity tests

| Species | No. of isolates tested |
|---|---|
| *Escherichia coli* O157: H7 ATCC 882364 | 1 |
| *E. coli* O157: H7 ATCC 43895 | 1 |
| *E. coli* O157: H7* | 1 |
| *Salmonella agona** | 1 |
| *S. anatis** | 1 |
| *S. choleraesuis** | 1 |
| *S. derby** | 4 |
| *S. enteritidis* CMCC 50335 | 1 |
| *S. london** | 1 |
| *S. saintoaul** | 1 |
| *S. typhimurium** | 2 |
| *Shigella boydii** | 1 |
| *Shigella dysenteriae** | 3 |
| *S. flexneri* CMCC 51061 | 1 |
| *S. flexneri** | 6 |
| *S. sonnei** | 1 |
| *E. coli* ATCC 8099 | 1 |
| *EPEC O111: K58B4** | 1 |
| *Bacillus proteus** | 1 |
| *B. subtilis** | 1 |
| *Citrobacter freundii** | 1 |
| *Pseudomonas aeruginosa** | 1 |
| *Staphylococcus albus** | 1 |
| *S. aureus* ATCC 6538 | 1 |
| *S. aureus* ATCC 25923 | 1 |
| *S. epidermidis** | 1 |
| *S. albus* ATCC 8032 | 1 |
| *S. faecalis* ACTT 32221 | 1 |
| *S. hemolyticus** | 1 |
| *Vibrio parahaemolyticus* ATCC 17802 | 1 |

ATCC, American Type Culture Collection; CMCC, National Center for Medical Culture Collection.
*Collection cultures of our laboratory (the Microbiology Laboratory in the Department of Medical Technology, West China School of Public Health, Sichuan University), identified by conventional biochemical and serological tests.

alternative. The objective of this study was to develop a novel magnetic capture–multiplex PCR (MP-PCR) assay for the simultaneous detection of *E. coli* O157: H7, *Salmonella* and *Shigella* in complex samples.

## Materials and methods
### Bacterial strains and selective enrichment broths

Forty-three bacterial strains (Tables 1 and 2) were tested in this study. Modified *E. coli* (mEC) broth (Beijing Land Bridge Technology Co. Ltd, Beijing, China) was used as the selective enrichment medium for *E. coli* O157: H7 (Chinese

**Table 2**   Capture efficiencies of MPs to different bacteria ($\bar{x} \pm$)

|  | MPs-bacteria (CFU mL$^{-1}$) | Supernatant-bacteria (CFU mL$^{-1}$) | Capture efficiencies (%) |
|---|---|---|---|
| *Escherichia coli* ATCC 8099 | $3.3 \times 10^4 \pm 2.6 \times 10^3$ | $6.2 \times 10 \pm 2.5 \times 10$ | 99.8 |
| *E. coli* O157: H7 ATCC 43895 | $1.9 \times 10^3 \pm 3.7 \times 10^2$ | $3.3 \pm 1.5$ | 99.8 |
| *E. coli* O157: H7 ATCC 882364 | $5.2 \times 10^3 \pm 3.8 \times 10^3$ | $4.0 \pm 2.6$ | 99.9 |
| *Salmonella enteritidis* CMCC 50335 | $3.1 \times 10^4 \pm 4.4 \times 10^3$ | $2.3 \times 10^2 \pm 3.0$ | 99.3 |
| *S. typhimurium** | $9.5 \times 10^4 \pm 4.4 \times 10^4$ | $1.6 \times 10^2 \pm 6.3 \times 10$ | 99.8 |
| *S. agona** | $4.0 \times 10^3 \pm 4.0 \times 10^2$ | $9.0 \pm 6.2$ | 99.8 |
| *S. derby** | $4.3 \times 10^3 \pm 1.1 \times 10^3$ | $6.7 \pm 0.6$ | 99.8 |
| *S. anatis** | $4.4 \times 10^4 \pm 7.5 \times 10^3$ | $2.7 \times 10 \pm 4.5$ | 99.9 |
| *Shigella dysenteriae** | $1.3 \times 10^4 \pm 5.0 \times 10^2$ | $4.3 \times 10 \pm 2.7 \times 10$ | 99.7 |
| *S. flexneri** | $5.3 \times 10^3 \pm 7.5 \times 10^2$ | $1.4 \times 10 \pm 1.7$ | 99.7 |
| *Pseudomonas aeruginosa** | $1.7 \times 10^4 \pm 2.4 \times 10^3$ | $6.5 \times 10 \pm 1.3 \times 10$ | 99.6 |
| *Bacillus cereous** | $3.8 \times 10^4 \pm 4.2 \times 10^3$ | $0.0 \pm 0.0$ | 100.0 |
| *Staphylococcus aureus* ATCC 6538 | $9.5 \times 10^4 \pm 8.5 \times 10^3$ | $5.7 \times 10 \pm 7.0$ | 99.9 |
| *S. albus* ATCC 8032 | $1.4 \times 10^5 \pm 1.6 \times 10^4$ | $1.0 \times 10^2 \pm 3.8 \times 10$ | 99.9 |
| *Streptococcus faecalis* ACTT 32221 | $1.5 \times 10^5 \pm 5.9 \times 10^4$ | $5.7 \pm 1.5$ | 100.0 |
| *Listeria monocytogenes** | $1.3 \times 10^4 \pm 8.4 \times 10^2$ | $3.2 \times 10 \pm 5.6$ | 99.8 |
| *Vibrio parahemolyticus* ATCC 17802 | $6.2 \times 10^3 \pm 1.1 \times 10^3$ | $1.8 \times 10^2 \pm 1.8 \times 10$ | 97.2 |

MP, magnetic particle; MPs-bacteria, the number of bacteria captured by the MPs; Supernatant-bacteria, the number of bacteria in the supernatant; ATCC, American Type Culture Collection; CMCC, National Center for Medical Culture Collection; CFU, colony-forming unit.
*Collection cultures of our laboratory (the Microbiology Laboratory in the Department of Medical Technology, West China School of Public Health, Sichuan University), identified by conventional biochemical and serological tests.

National Standard, 2008a), and *Salmonella–Shigella* (SS) enrichment broth (Hangzhou Microbial Agent Co. Ltd, Hangzhou, China) was used for the simultaneous selective enrichment of SS.

## Preparation of MPs

FeCl$_2$ and FeCl$_3$ were used to prepare the MPs. Briefly, 3 g FeCl$_2$.4H$_2$O (Jinhui Chemical Reagent Co. Ltd, Tianjin, China) and 8.2 g FeCl$_3$.6H$_2$O (Jinhui Chemical Reagent Co. Ltd, Tianjin, China) were each dissolved in 50 mL H$_2$O and filtered. The two solutions were mixed together, treated by ultrasonication (40 kHz), and stirred (120 times min$^{-1}$) for 15 min. Alkali was added with continuous stirring until a pH of 11 was achieved. The resulting MPs were collected using an external magnetic separator (Shanghai ZJ Bio-tech Co. Ltd, Shanghai, China).

## Capture efficiencies of MPs for different bacteria

The capture efficiencies of the MPs were verified using an aerobic plate count method. Following preliminary experiments to optimize the MP volume and concentration, and adsorption time and temperature (50 μL of 100 μg μL$^{-1}$; 15 min; 20 °C, respectively), various target and non-target bacteria were individually tested with the MPs. Briefly, for each target or non-target bacterium, a 50 μL volume of MPs

(100 μg μL$^{-1}$) was added to a 1-mL solution of the bacterium. The solution was gently shaken at room temperature for 15 min, and then subjected to a magnetic field. The bacterial cells captured by the MPs [resuspended in 1 mL normal saline (NS) ] and those remaining in the supernatant (1 mL) were separately serially diluted in NS, 1 mL volumes of the $10^0$–$10^2$ NS dilutions of each series were plated onto Luria broth (LB) agar plates, and the plates were incubated overnight at $36 \pm 1$°C. For each series, the number of colony-forming units (CFUs) was counted on plates having 30–300 colonies. The number of bacteria in the supernatant or bound to the MPs was calculated as the number of CFUs multiplied by the dilution factor. The capture efficiency was defined as the ratio of the number of target bacteria captured by the MPs to the total number of colonies, according to the formula:

$$\text{capture efficiency} = \sum C_n \Big/ \sum (C_n + S_n),$$

Where, n is the number of repeated experiments, and *C* and *S* are the numbers of bacteria captured by the MPs and remaining in the supernatant, respectively. The experiments were repeated three times.

## PCR template preparation

Genomic DNA template was prepared from 1.0 mL of each target bacterium using a simple boiling method with the addition of bovine serum albumin (BSA; Amresco, Solon, USA). In

**Table 3** Primers used in the multiplex polymerase chain reaction

| Primer | Sequence (5′ to 3′) | Target gene | Amplicon size (bp) |
|---|---|---|---|
| *rfbE*-f | CACGAAAACGTGAAATTGCTGATATT | *rfbE* | 169 |
| *rfbE*-r | TCGATGAGTTTATCTGCAAGGTGATTC | | |
| *invA*-f | GTGAAATTATCGCCACGTTCGGGCAA | *invA* | 284 |
| *invA*-r | TCATCGCACCGTCAAAGGAACC | | |
| *ipaH*-f | GTTCCTTGACCGCCTTTCCGATACCGTC | *ipaH* | 620 |
| *ipaH*-r | GCCGGTCAGCCACCCTCTGAGAGTAC | | |

brief, the bacterial cells in 1.0 mL of an enrichment culture (mEC broth for *E. coli* O157: H7; SS broth for *Salmonella* and *Shigella*; culture conditions: 36 ± 1°C, 8 h, with shaking) were washed twice by centrifugation for 2 min at 15 000 rpm and resuspension in 1 mL NS. A 50 μL volume of MPs (100 μg μL$^{-1}$) was added and the mixture was gently shaken at room temperature for 15 min. The MP–bacterial complexes were separated using an external magnetic separator, resuspended in a final volume of 300 μL Tris – Ethylene Diamine Tetraacetic Acid (TE) buffer (Trevanich *et al*., 2010) plus 30 μL BSA (100 μg μL$^{-1}$), and boiled for 10 min. The supernatant was used as the PCR template after brief centrifugation.

### Primers

The primer sequences used in this study are shown in Table 3. Primers targeting the *ipaH* gene of *Shigella* and the *invA* gene of *Salmonella* were synthesized as described previously (Rahn *et al*., 1992; Malorny *et al*., 2003; Li & Mustapha, 2004). Primers targeting the *rfbE* gene of *E. coli* O157: H7 were designed using Primer Premier 5.0 software (PREMIER Biosoft Int., California, USA).

### Optimization of the multiplex PCR assay

The multiplex PCR assay was performed in a DNA thermal cycler S1000 (Bio-Rad, Singapore). A total volume of 50 μL of PCR mixture contained 25 μL from the Multiplex PCR Mix 1 (TaKaRa, Dalian, China), primers (0.8 μM of each of *rfbE*-f and *rfbE*-r, 0.6 μM of each of *invA*-f and *invA*-r and 0.75 μM of each of *ipaH*-f, and *ipaH*-r; Invitrogen), 0.25 μl Multiplex PCR Mix 2 (TaKaRa, Dalian, China), 5 μL of pathogen DNA mix from the three pathogens and sterile distilled water. A negative control was included containing 5 μL of distilled water instead of the DNA template. The conditions for multiplex PCR involved a preheating step of 95 °C for 5 min followed by 35 cycles of 95 °C for 50 s, 60 °C for 1 min and 72 °C for 45 s, and a final hold at 72 °C for 7 min. The concentration ratios of the PCR primers and the thermal

cycling program were optimized. Following amplification, the PCR products were subjected to gel electrophoresis (90 V, 30 min) in 2.0% agarose, and the bands were visualized by staining with GoldView I (Solarbio, Shanghai, China).

### Sensitivity and specificity of the assay

The sensitivity of the detection system was defined as the lowest viable count of the subject pathogen that yielded the desired PCR product. For sensitivity experiments with pure cultures, an overnight culture of a target bacterium was suspended in NS to a MacFarland standard of 1 (equal to $3 \times 10^8$ CFU of *E. coli*) and serially diluted. DNA template extracted from the suspension was subjected to multiplex PCR. To determine the cell density of the target bacterium, 10-fold serial dilutions were incubated overnight at 36 ± 1°C on three replicate LB agar plates. The detection system was also used to detect the target bacteria in fecal and food samples spiked with various bacterial concentrations. Firstly, human feces and food samples were confirmed to be free of *Salmonella*, *Shigella* and *E. coli* O157: H7 using conventional culture methods (Huang, 2006) and the multiplex PCR assay. The fecal samples (about 0.1 g) were then artificially spiked with various concentrations ($10^0$–$10^2$ CFU) of target bacteria and incubated in 10 mL of selective enrichment broth (mEC broth for *E. coli* O157: H7 and SS for broth *Salmonella* and *Shigella*) for 8 h at 36 ± 1°C with shaking. For food samples, chicken, cucumbers and cooked rice (25 g per sample) were inoculated with different concentrations of target bacteria (final concentration: $10^0$ to $10^2$ CFU g$^{-1}$), then incubated in 225 mL broth as described previously. Following incubation, the genomic DNA was extracted from 1 mL enrichment broths as described previously and subjected to multiplex PCR. The aerobic plate count method was used to determine the number of bacteria per mL. The specificity of the assay was determined by separately testing a panel of 41 bacterial species (Table 1) as templates in the reaction. Experiments were conducted in triplicate.

### Stool sample analysis

The magnetic capture–multiplex PCR platform was compared with conventional culture assay (Huang, 2006) and multiplex PCR without the MP-based magnetic capture component. Eighteen stool samples each artificially inoculated with a target bacterium (6 *Salmonella*, 6 *Shigella* and 6 *E. coli* O157: H7) and six uninoculated controls were analyzed using each of the three assays. DNA extraction for multiplex PCR without the MP-based magnetic capture

component was as follows: bacterial cells from 1 mL of enrichment culture (mEC broth for *E. coli* O157: H7 and SS broth for each of *Salmonella* and *Shigella*; culture conditions: $36 \pm 1°C$, 8 h, with shaking) were washed twice with NS by centrifugation for 2 min at 15 000 rpm and resuspension in 330 µL TE, then boiled for 10 min. After brief centrifugation, the supernatant was used as the PCR template. The magnetic capture–multiplex PCR assay was also applied to 192 stool specimens collected from West China Women's and Children's Hospital and local Centers for Disease Control (CDC), and the results were compared with those using conventional culture methods.

## Food sample analysis

To demonstrate the applicability of the magnetic capture–multiplex PCR assay to food samples, ground beef, chicken and vegetables were artificially inoculated with 100 µL of the target pathogens to achieve inoculation levels of approximately $10^2$ CFU $g^{-1}$. Negative controls were prepared by adding 100 µL of NS to uninoculated food samples. Subsamples of each food sample (25 g) were transferred into 225 mL of each of SS broth and mEC broth, homogenized for 2 min and incubated at $36 \pm 1°C$ for 8 h with shaking. Then the DNA was extracted and used for multiplex PCR amplification under optimal conditions (as described above). The results were compared with those of conventional culture methods (Chinese National Standard 2003, 2008a, 2008b). Food samples (25) purchased from local food markets were also analyzed using the MP assay and conventional culture methods.

## Results

### Capture efficiency of MPs

Assessment of the capture efficiency of the MPs included the target bacteria, Gram positive bacteria, Gram negative bacteria and indicator bacteria. The MP capture efficiencies for most bacteria exceeded 95% (Table 2). High MP capture efficiencies for bacteria at varying concentrations ($10^1$–$10^6$) were also observed (data not shown).

### Optimized multiplex PCR conditions

A multiplex PCR assay was established for the simultaneous identification of target bacteria. Comparison of various reaction conditions enabled the selection of appropriate concentrations of the *rfbE*, *invA* and *ipaH* primers (1.80, 0.9
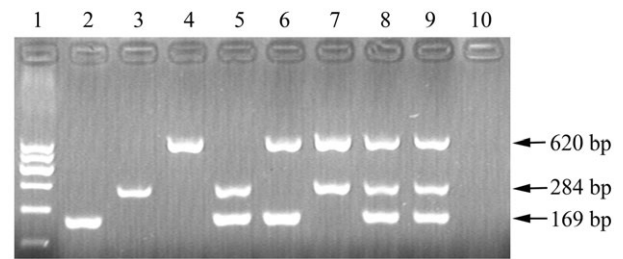


**Figure 1** Agarose gel electrophoresis of multiplex polymerase chain reaction (PCR) products from the DNA of *Escherichia coli* O157: H7, *Salmonella* and *Shigella*. Lanes: 1, PCR markers (600, 500, 400, 300, 200 and 100 bp, respectively); 2, *E. coli* O157: H7; 3, *Salmonella*; 4, *Shigella*; 5, *E. coli* O157: H7 + *Salmonella*; 6, *E. coli* O157: H7 + *Shigella*; 7, *Salmonella* + *Shigella*; 8–9, *E. coli* O157: H7 + *Salmonella* + *Shigella*; 10, sterile water (negative control).

and 0.56 µM, respectively). The optimized thermal protocol for multiplex PCR was: 5 min at 95 °C followed by 35 cycles of 50 s at 95 °C, 60 s at 63 °C, 45 s at 72 °C, and a final extension for 7 min at 72 °C. Under these conditions the multiplex PCR assay yielded a 169 bp DNA product from *E. coli* O157: H7, a 284 bp product from *Salmonella enteritidis*, and a 620 bp product from *Shigella flexneri* (Figure 1). When the DNA extracted from multiple target organisms were combined in the same reaction, two or three corresponding amplicons of different sizes were observed, respectively.

### Sensitivity and specificity

For pure cultures of the target bacteria diluted in NS, the sensitivity of the multiplex assay was $8.7 \times 10^3$ CFU $mL^{-1}$ for *Salmonella*, $3.3 \times 10^3$ CFU $mL^{-1}$ for *Shigella* and $8.3 \times 10^3$ CFU $mL^{-1}$ for *E. coli* O157: H7. The sensitivity following incubation for 8 h was $10°$CFU $g^{-1}$ in food ($7.0 \pm 0.9 \times 10°$CFU $g^{-1}$ for *Salmonella*, $4.0 \pm 0.7 \times 10°$CFU $g^{-1}$ for *Shigella* and $8.1 \pm 0.9 \times 10°$CFU $g^{-1}$ for *E. coli* O157: H7) and 1–10 CFUs per stool sample ($9.5 \pm 0.6 \times 10°$CFU for *Salmonella*, $3.3 \pm 0.9 \times 10°$CFU for *Shigella* and $8.1 \pm 0.8 \times 10°$CFU for *E. coli* O157: H7; Figure 2). For specificity experiments, the MP-PCR assay showed 100% selectivity (inclusion and exclusion) in detecting the panel of 41 target and non-target bacterial strains (Table 1).

### Stool sample analysis

The MP-PCR assay was compared with conventional culture methods for the detection of the target bacteria. The bacteria
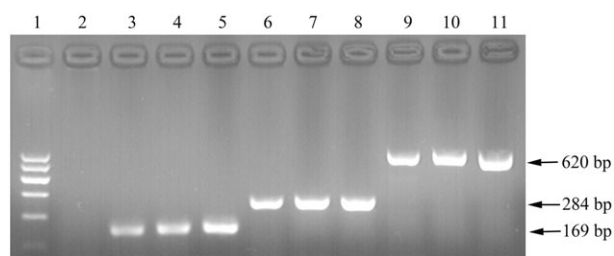
**Figure 2** Sensitivity of the molecular assay in stool sample analysis. Lanes: 1, polymerase chain reaction markers (600, 500, 400, 300, 200 and 100 bp, respectively); 2, sterile water (negative control); 3–5, stool samples inoculated with *Escherichia coli* O157: H7 [$10^0$–$10^2$ colony-forming units (CFU) per sample, respectively]; 6–8, stool samples inoculated with *Salmonella* ($10^0$–$10^2$ CFU per sample, respectively); 9–11, stool samples inoculated with *Shigella* ($10^0$–$10^2$ CFU per sample, respectively).
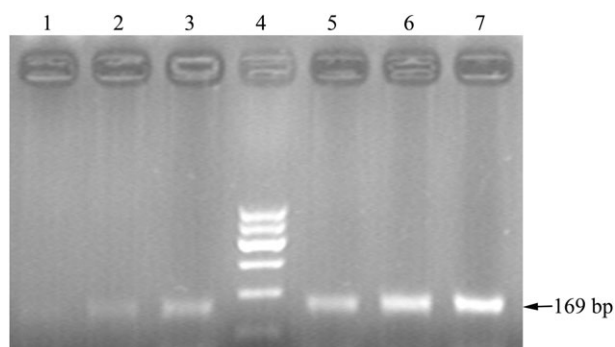


**Figure 3** Comparative analysis of *Escherichia coli* O157: H7 in stool samples using the established assay and multiplex polymerase chain reaction (PCR) without MP-based magnetic capture. Lanes: 1–3, amplification of *E. coli* O157: H7 using multiplex PCR without magnetic particles (MP)-based magnetic capture [$10^0$–$10^2$ colony-forming units (CFU) per sample, respectively]; 4, PCR markers (600, 500, 400, 300, 200 and 100 bp, respectively); 5–7, amplification of *E. coli* O157: H7 using multiplex PCR with MP-based magnetic capture ($10^0$–$10^2$ CFU per sample, respectively).

in all 18 of the artificially spiked samples were correctly identified by both approaches, and none were detected in the six negative controls. Amplification by multiplex PCR with and without MPs indicated that the brightness of the amplification bands from multiplex PCR with MPs was greater than that from multiplex PCR without MPs, and indicated that the former assay might provide better results than that of the later one (Figure 3). Of the 192 samples collected from West China Women's and Children's Hospital and local CDCs, four were identified as *Salmonella* and one was identified as *Shigella* by both the established assay and conventional culture methods.
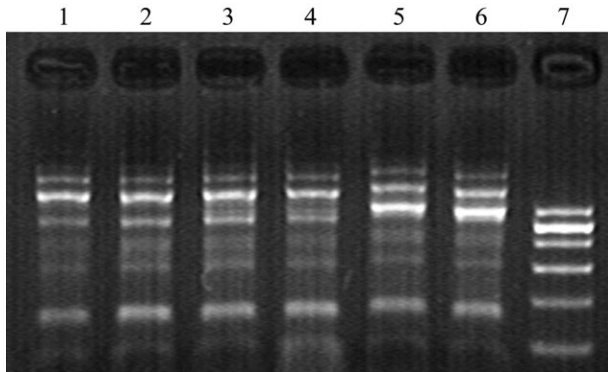
## Food sample analysis

The MP–multiplex PCR method detected the pathogens specifically and accurately in the artificially inoculated food samples. Among the 25 food samples collected from local food markets, one (chicken) was found to contain *Salmonella* by both the MP assay and conventional culture methods.

## Discussion

In this study, we established a new platform based on the use of MPs to separate and concentrate pathogenic bacteria from complex samples. Coupled with multiplex PCR, this enabled the simultaneous detection of *E. coli* O157: H7, *Salmonella* and *Shigella*. The assay was applied with high specificity and sensitivity to analysis of both fecal and food samples. The capture efficiency of MPs is critical if high detection sensitivity is to be achieved, and results indicated that our lab-made MPs had excellent capture performance to bacterial cells. In the stool sample analyses, the high sensitivity of the multiplex PCR and the 100% detection rate also indicated that the capture efficiency of the MPs was very effective for detecting the target pathogens in complex samples. The introduction of MPs into the detection platform provides other advantages. Compared with antibody-modified magnetic beads, the MPs were very cheap to produce and could be prepared very easily from $FeCl_2$ and $FeCl_3$. Moreover, the storage and reaction conditions for the MPs without antibody were not strict, and the absorption of the MPs to bacteria was not limited by the type of antibody coupled to the MPs. As bacterial foodborne diseases can be caused by one or several kinds of unknown pathogenic bacteria, the use of MPs offers a promising approach to the simultaneous separation of different pathogens in complex samples, and could have application for large-scale surveillance programs and routine detection.

The boiling method was selected for DNA template preparation. This is a simple, rapid and cost-effective procedure for DNA preparation requiring no sophisticated equipment and has been widely used in the detection of a variety of pathogenic microorganisms (Trkov *et al*., 1999; Medici *et al*., 2003; Trevanich *et al*., 2010). However, in preliminary experiments to extract DNA from target bacteria captured by MPs we observed precipitation and coagulation of the MPs during the boiling process, and there was a marked decrease in PCR products obtained, where this phenomenon occurred (data not shown). One explanation for these observations is that the MPs precipitated and coagulated during heating, resulting in some of the captured bacteria and

**Figure 4** Stool sample analysis using multiplex polymerase chain reaction (PCR) for the detection of pathogens in food. Lanes 1–6, PCR products of stool samples (lines 1–2, stool sample A; lines 3–4, stool sample B; lines 5–6, stool sample C); lanes 7, PCR markers (600, 500, 400, 300, and 200 bp respectively).

released DNA might subsequently be warped into the sediment, which led to fewer PCR amplification products. In addition, iron is the main component of the MPs, which might be released during the boiling procedure, and could affect PCR amplification (Bockstahler _et al._, 1997). These problems may explain why no previous study has reported the combination of MPs and PCR for pathogen detection. We found that the introduction of BSA into the boiling process facilitated the suspension of bead–bacteria complexes, and significantly improved the detection sensitivity. BSA has previously been used as a competitive binding agent to release attached spores from MPs (Yitzhaki _et al._, 2006), and as an effective and widely used additive to overcome inhibitory effects in PCR processes (Höss _et al._, 1992; Akane _et al._, 1993). This protein has also been found to be capable of scavenging iron, and thereby preventing its binding to and inactivation of Taq DNA polymerase (Kreader, 1996; Primorac, 2004). These findings may explain the effectiveness of BSA in our assay.

The primer sets initially selected for use in the MP-PCR assay were designed to detect pathogens in cider samples (Li & Mustapha, 2004). However, in this study, their application to stool samples, which are characterized by high background microflora levels and the presence of various PCR inhibitors, resulted in nonspecific banding that made it difficult to distinguish the pathogen-specific bands (Figure 4). To overcome this problem, we changed the primer sets used and further optimized the PCR reaction conditions. In brief, for the detection of _E. coli_ O157: H7 primers for the _rfbE_ gene replaced those for the _uidA_ gene (Al-Ajmi _et al._, 2006; Carlson _et al._, 2009). We retained use of the _invA_ and _ipaH_ genes for _Salmonella_ and _Shigella_, respectively, because of

their good specificity (Malorny _et al._, 2003; Wang _et al._, 2010). All 2000 _Salmonella_ serovars tested so far apparently contain _inv_ genes, which code for invasion virulence factors in those bacteria (Chiu & Ou, 1996), and the _ipaH_ gene is almost exclusively restricted to the four _Shigella_ spp. in the Asian region (Seidlein _et al._, 2006). However, new primers were designed for the _invA_ gene of _Salmonella_ to give amplification products of 284 bp rather than 398 bp. Under the optimized conditions, nonspecific banding was eliminated (Figure 2) and the target bands could be easily identified. Results indicating 100% selectivity (both inclusion and exclusion) were obtained when the MP-PCR assay was applied to the test strains listed in Table 1.

The detection of pathogens in stools is of significance for public health. To assure food safety, the Chinese food service staffs involved in food production activities are required to take regular monitoring for pathogenic organisms. However, conventional culture methods are still used for routine detection of these bacteria in China, and these techniques are time consuming and labor intensive. Alternatives based on DNA detection have been established. However, stool samples are typically characterized by low numbers of pathogenic bacteria, high numbers of normal gut microflora and the presence of many complex inhibitors, all of which potentially limit the sensitivity and specificity of molecular-based detection methods (Gillers _et al._, 2009). In our study, we used MPs to separate and concentrate the bacteria from stool samples, decreasing the effect of inhibitors and providing better results than in the absence of MP absorption. As non-target bacteria in feces could compete with target bacteria for attachment to the MPs, a selective enrichment step was included in the detection assay. Following an 8 h enrichment step, we were able to detect target bacteria at a contamination level of 1–10 CFUs per stool sample. Comparison of results of the MP-PCR assay with those based on conventional culture methods confirmed the ability of the former to more rapidly detect the target bacteria. Further, the established assay was applied to food sample, and good results were obtained. These findings indicated that this MP-PCR assay might be applicable to other samples, especially those containing impurities that make detection difficult by multiplex PCR without pretreatment.

In conclusion, we established a novel assay combining nonspecific MPs with multiplex PCR for the simultaneous separation of _E. coli_ O157: H7, _Salmonella_ and _Shigella_. This assay was effective in detection of pathogens in complex samples, and provides a promising method for pathogen detection in food poisoning outbreaks, and in the routine surveillance work of public health agencies.

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