International Journal of Nanomedicine

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ORIGINAL RESEARCH

A rapid and highly sensitive protocol for the detection of *Escherichia coli* O157:H7 based on immunochromatography assay combined with the enrichment technique of immunomagnetic nanoparticles

This article was published in the following Dove Press journal:
International Journal of Nanomedicine
28 November 2011
Number of times this article has been viewed

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Background: *Escherichia coli* O157:H7 (*E. coli* O157:H7) is an important pathogenic bacterium that threatens human health. A rapid, simple, highly sensitive, and specific method for the detection of *E. coli* O157:H7 is necessary.

Methods: In the present study, immunomagnetic nanoparticles (IMPs) were prepared with nanopure iron as the core, coated with *E. coli* O157:H7 polyclonal antibodies. These IMPs were used in combination with immunochromatographic assay (ICA) and used to establish highly sensitive and rapid kits (IMPs+ICA) to detect *E. coli* O157:H7. The kits were then used to detect *E. coli* O157:H7 in 150 food samples and were compared with conventional ICA to evaluate their efficacy.

Results: The average diameter of IMPs was 56 nm and the amount of adsorbed antibodies was 106.0 μg/mg. The sensitivity of ICA and IMPs+ICA was 10^5 colony-forming units/mL and 10^3 CFUs/mL, respectively, for purified *E. coli* O157:H7 solution. The sensitivity of IMPs+ICA was increased by two orders, and its specificity was similar to ICA.

Conclusion: The kits have the potential to offer important social and economic benefits in the screening, monitoring, and control of food safety.

Keywords: colloidal gold, immunomagnetic nanoparticles, *Escherichia coli* O157:H7, immunochromatographic assay

Introduction

Enterohemorrhagic *Escherichia coli* O157:H7 is a new enteropathogenic bacterium that can cause human diarrhea, hemorrhagic enteritis, thrombotic thrombocytopenic purpura, and hemolytic uremic syndrome. Since the USA reported food poisoning caused by this strain of bacteria in 1982,[1] multiple epidemic outbreaks have occurred throughout the world. It has been estimated that 73,000 individuals are infected by *E. coli* O157:H7 per year in the USA. Of those 73,000 individuals, approximately 62,000 are infected through food transmission and 11,000 through water transmission.[2] Since the first case of infection was reported in Xuzhou, China in 1986,[3] *E. coli* O157:H7 has been successively isolated from humans, livestock, and other animals in Fujian, Gansu, Zhejiang, Jiangsu, and Anhui. The threat of this pathogenic bacterium is growing in China.[4] Improving protocol for the detection of *E. coli* O157:H7 in animal food and the environment will play an important role in

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http://dx.doi.org/10.2147/IJN.S25684

epidemiological investigation and the prevention and control of this disease. The method of conventional isolation and culture takes a few days to produce results. There are many methods for the detection of *E. coli* O157:H7, including polymerase chain reaction, gene chip, phage typing, biosensor technique, and enzyme-linked immunosorbent assay.[5–7] Most of these methods, however, require special equipment and a long detection time.

Immunochromatographic assay (ICA) has been widely used in biological detection, including for a variety of pathogenic microorganisms. ICA is simple, rapid, highly sensitive, specific, and does not require special equipment or reagents. The results of ICA can be judged by the naked eye and are readily preserved. However, false negatives occur easily in ICA, and the sensitivity of ICA is generally less than 1×10^5 colony-forming units (CFUs)/mL.[8]

Immunomagnetic nanoparticles (IMPs) enrichment is an advanced tool for detecting pathogenic organisms, and IMPs are characterized by high specificity, ability to form high-concentration suspensions, high separation rates, and non-influence on organism activity. In this study, a new, modified IMPs were prepared with nanopure iron (Fe) as the core, coated with *E. coli* O157:H7 polyclonal antibodies, in combination with ICA technology. Sensitivity increased by two orders. The protocol was used to detect *E. coli* O157:H7 in 150 food samples, including milk, purified water, and beef, and was compared with conventional ICA to evaluate its advantages and disadvantages.

Materials and methods

Preparation of IMPs with *E. coli* O157:H7

One hundred milligrams of sodium alginate (Sigma-Aldrich, St Louis, MO) solution was dissolved in 4 mL of water. Then, 2 mL of 5% pure Fe magnetic fluid solution was added (average diameter 10 ± 5 nm, purity 99.99%, specific saturation magnetization ≥1800 Am^2/kg, intrinsic coercivity ≥34.8 KA/m; provided by Shenzhen Junye Nano Material Co Ltd, Shenzhen, China). The magnetic nanoparticles were prepared and samples vacuum freeze-dried for storage as previously described.[9]

One milligram of prepared magnetic nanoparticles was taken out and washed three times with phosphate buffer solution (PBS). Then, 0.01 mol/L PBS (pH 7.0) was added to the final 4 mL volume 5 mg carbodiimide (Sigma-Aldrich, St Louis, MO), and 7.5 mg sulfo-N-hydroxysuccinimide (Sigma-Aldrich) was added and mixed thoroughly for 15 minutes at room temperature. A total of 50 mg of 6-aminocaproic acid was then added and stirred for 3 hours. Following this, 2 μg of *E. coli* O157:H7 polyclonal antibodies (Abcam, Cambridge, MA) was added and stirred for 1 hour. The mixture was then sealed with 1 mL of 0.2 mol/L glycine solution containing 0.2% bovine serum albumin (BSA) (Gibco, Carlsbad, CA). The mixture was preserved at 4°C followed by magnetic separation and addition of storage solution. The morphology was observed with a transmission electron microscope (TECHAI-10; Philips, Amsterdam, The Netherlands) and light microscope (TE2000-U; Nikon, Tokyo, Japan).

Preparation of colloidal gold

Colloidal gold was prepared as previously described by Frens.[10] HAuCl (100 mL, 0.01% [W/V]) (Sigma-Aldrich) was heated to boiling, then 5 mL trisodium citrate (1% [W/V]) (Sigma-Aldrich) was rapidly added while the mixture was stirred at high speed and heated for 30 minutes. After natural cooling, colloidal gold was filtered through a 0.22 μm membrane and stored in the dark at 4°C for future use.

Preparation of immune colloidal gold

Choice of optimal protein content

E. coli O157:H7 monoclonal antibodies (ViroStat, Portland, ME) were diluted to 4 μg/mL, 6 μg/mL, 8 μg/mL, 10 μg/mL, 12 μg/mL, 14 μg/mL, 16 μg/mL, and 18 μg/mL with 0.01 M pH 7.4 PBS. *E. coli* O157:H7 monoclonal antibodies of different concentrations (1 mL) were each added into 1 mL of colloidal gold, and another 1 mL of colloidal gold without protein served as control. After 5 minutes, this was added to 0.1 mL 10% NaCl. Two hours later, the concentration that stabilized the colloidal gold, plus 20% was served as the optimal protein-labeled content.

Choice of optimal pH

Colloidal gold (1 mL) was then added into each of eight test tubes and these were adjusted to pH values of 3, 4, 5, 6, 7, 8, 9, and 10 with K_2CO_3 (0.1 mol/L). *E. coli* O157:H7 monoclonal antibodies of optimal protein content were added and mixed. After 5 minutes, 0.1 mL 10% NaCl was added. Two hours later, test tubes were observed to determine the optimal pH value.

Colloidal gold labeled with antibodies

Colloidal gold (1 mL) was adjusted to pH 8.0 followed by the addition of *E. coli* O157:H7 monoclonal antibodies (16.8 μg/mL) with stirring for 2–3 minutes. It was sealed with 10% BSA. The mixture was then centrifuged at 1500 r/min

for 20 minutes at 4°C. After the removal of sediment, the supernatant was centrifuged at 10,000 r/min for 30 minutes at 4°C. The sediment was resuspended with TBS (0.005 mol/L, pH 7.6, 0.1% BSA, and 0.05% sodium azide) and then stored at 4°C for future use.

Preparation of ICA to *E. coli* O157:H7

Preparation of the colloidal gold-antibody pads

The colloidal gold-labeled antibody was diluted 1:1, 1:1.5, and 1:2 with PBS (0.01 mol/L) and then placed on fiberglass membrane (GF-06; Gold Bio-Pharmaceutical Technology Co, Ltd, Shanghai, China) at 37°C for 30 minutes. When other conditions were unchanged, phalanx titrimetry was performed to determine the optimal dilution.

Concentrations of polyclonal antibody for test line and rabbit anti-mouse IgG for control line

E. coli O157:H7 polyclonal antibodies and rabbit antimouse immunoglobulin G (IgG) (Tingguo, Beijng, China) were diluted with PBS (0.01 mol/L, pH 7.6) and spotted onto nitrocellulose (NC) membrane (Hi-Flow Plus HF135; EMD Millipore, Billerica, MA) with different flow rates to be served as test (T) line and control (C) line. After drying at room temperature, immunochromatographic strips were made. ICA was performed with a pure culture of *E. coli* O157:H7 as antigens to determine the optimal concentrations according to coloration and chromatographic velocity.

Assembly of ICA to *E. coli* O157:H7

The NC membrane containing *E. coli* O157:H7 polyclonal antibodies and rabbit anti-mouse IgG secondary antibodies, absorption pad, colloidal gold-labeled antibody pad (glass-fiber membrane), and sample pad (absorbent paper) (Gold Bio-Pharmaceutical Technology Co, Ltd) were assembled to form the strip and attached to a plastic scaleboard. The assembly was cut into strips 60 mm long and 3 mm wide. The ICA for detection of *E. coli* O157:H7 was obtained.

Characteristics of ICA to *E. coli* O157:H7

Specificity of ICA to *E. coli* O157:H7

The specificity of ICA was observed in *E. coli* of ten different serotypes and non-*E. coli* of eleven different strains. Each strain was incubated in Luria-Bertani (LB) broth (HKM, Guangzhou, China) (*E. coli* O157:H7 EDL933; *E. coli* O157:H7 86–24; *E. coli* O157:H7 [F25]; *E. coli* O157:H6p; *E. coli* O26:H11; *E. coli* O148:H28; *E. coli* O3:H2; *E. coli* O15:H$^-$; *E. coli* O103:H$^-$; *E. coli* LE392), Selenite Cystine broth (HKM, Guangzhou, China) to increase bacterium fluid (*Salmonella typhi*, *Salmonella paratyphi* A, and *S. paratyphi* B), 3.5% NaCl sodium chloride violet purple enrichment (*Vibrio parahaemolyticus*), 7.5% NaCl sodium chloride broth (*Staphylococcus aureus* and *Staphylococcus epidermidis*), glucose-increased bacterium fluid (β *Streptococcus*, *Streptococcus pneumoniae*, and *Enterococcus faecalis*), and glycerin broth (*Malleomyces* and *Burkholderia pseudomallei*) at 37°C overnight. After inactivation at 100°C for 15 minutes, 250 μL from each inactivated liquid was placed into a 1.5 mL centrifuge tube. The ICA was soaked in the inactivated liquid, and 10 minutes later the results were observed.

Sensitivity of ICA to *E. coli* O157:H7

After incubating in LB broth at 37°C overnight, the *E. coli* O157:H7 medium was centrifuged and resuspended with PBS (0.01 mol/L, pH 7.4). *E. coli* O157:H7 was adjusted to 10^3–10^8 CFUs/mL. After inactivation at 100°C for 15 minutes, 250 μL from each concentration solution was placed into a 5 mL centrifuge tube, then the ICA was soaked in the solution. Ten minutes later, the results were observed.

Repetitiveness of ICA to *E. coli* O157:H7

Strips from different batches were used to detect two positive samples and two negative samples, and each sample was detected four times to observe the repetitiveness.

Stability of ICA to *E. coli* O157:H7

These strips were stored either at room temperature or 4°C. The strips at room temperature and 4°C were detected with strong positive, weak positive, and negative samples every 30 days.

Detection of IMPs combined with ICA

IMPs enriched for *E. coli* O157:H7

E. coli O157:H7 was diluted 1:10 with sterile saline. One milliliter of bacillus solution from different concentrations (10^3–10^6 CFUs/mL) was placed into Eppendorf tubes. IMPs and *E. coli* O157:H7 (10 μL, 30 μL, 50 μL, 70 μL, 100 μL) were also added into the EP tubes and mixed at room temperature for 10 minutes, separated using a magnetic separator before removal of supernatant, and washed twice to obtain the extracts containing *E. coli* O157:H7. The IMPs enriched for *E. coli* O157:H7 were resuspended with 0.1 mL of PBS, placed in a 60°C water bath for 15 minutes, and then underwent magnetic separation. The supernatant contained the enriched *E. coli* O157:H7.

Detection of IMPs combined with ICA to *E. coli* O157:H7

Sensitivity

IMPs were used to gather *E. coli* O157:H7 in 10^2–10^8 CFUs/mL bacillus solutions. After inactivation at 100°C for 15 minutes, the ICA was soaked in the bacillus solutions. Ten minutes later, the results were observed.

Specificity

IMPs were used to gather *E. coli* of ten different serotypes and non-*E. coli* of eleven different strains. The obtained enrichment bacteria were resuspended with sterile PBS and then cultured in chromogenic *E. coli* O157:H7 agar (HKM) at 37°C for 24 hours to observe whether specific bacterium colonies would occur.

Detection of IMPs combined with ICA in food samples

Food sample

There were 50-aliquot samples each of milk, purified water, and beef. Each aliquot (10 mL) was added to 90 mL of sterile saline, following centrifugation at 8000 r/min for 1 minute, and was made into a 1:10 homogeneous dilution. *E. coli* O157:H7 strains were made into 10^7–10^5 CFUs/mL bacillus solutions. A 0.9 mL food sample (45 milk, 45 purified water, and 45 beef) was contaminated with 0.1 mL of *E. coli* O157:H7 (10^7–10^5 CFUs/mL) (each concentration contaminated 15 food samples) to give the final concentrations of the solution as 10^5–10^3 CFUs/mL. The other 15 food samples (five milk, five purified water, and five beef) were used as negative control.

Detection of *E. coli* O157:H7

IMPs (30 μL) were added into each contaminated food sample (1 mL) with rocking for 10 minutes, then separated using a magnetic separator. The gathering *E. coli* O157:H7 was incubated in a 60°C water bath for 15 minutes. The ICA was soaked in the medium. Ten minutes later, the results were observed.

Results and discussion

Characteristics of IMPs

When viewed under a transmission electron microscope, the IMPs were of regular spherical shape, showed good dispersion properties, and had a mean diameter of 56 nm (ranging from 34 nm to 86 nm). The antibody cross-linked on the surface of magnetic microspheres usually does not have full contact between the biological macromolecules and ligands as a result of steric hindrance, reducing the space utilization of the ligands. Therefore, in this study, a spacer arm of 6-amino hexanoic acid was linked with a carbon chain length of six covered with active groups to the microspheres. Following coupling to the corresponding antibodies using EDC, high-quality IMPs were successfully prepared.[11] There were up to 108.6 μg antibodies per milligram of IMPs, and flow cytometry showed that 97.4% of the IMPs possessed immunoactive antibodies. Large IMPs do not necessarily have a good separation effect and may produce false positive results. IMPs with a mean diameter of 50 nm can obtain the best separation effect.

Preparation of colloidal gold

Transmission electron microscope and a laser light scattering instrument showed that uniform colloidal gold particles had an average diameter of 20 ± 3 nm. The diameter of colloidal gold particles is strongly associated with the amount of trisodium citrate. When other conditions are unchanged, different amounts of trisodium citrate may obtain colloidal gold particles of different color and size.

Preparation of immunecolloidal gold

The minimum protein content of stable colloidal gold was 14 μg/mL. On this basis, 14 μg/mL plus 20% was used as the actual protein-labeled content (16.8 μg/mL). When the protein-labeled content was 16.8 μg/mL and the pH of colloidal gold was 7.0–9.0, immunecolloidal gold was stable and brightly colored with a pH of 8.0. Nonmodified colloidal gold is unstable in the high-concentration salt solutions due to the layer of negative charge around colloidal gold formed by residual anion in solution.[12] The surface of colloidal gold is modified with antibodies to make colloidal gold stable in high-concentration salt solution so coagulation cannot occur.

Preparation of ICA

The concentration of rabbit anti-*E. coli* O157:H7 polyclonal antibody serving as T line was identified as 1.0 mg/mL, and the concentration of rabbit antimouse IgG antibody serving as C line was identified as 5.0 mg/mL. NC membrane was coated by an automatic spray dot instrument (XYZ-3000D; BioDot, Irvine, CA) with the parameters 1 μL/cm and 5 cm/s, following drying, and was then sealed with PBS (0.01 mol/L, pH7.6, 1% BSA). The background was clear after ICA. Whether preparation of ICA is successful depends on the characteristics of antibodies. In this study, *E. coli* O157:H7 monoclonal antibody protein was identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and results indicated highly pure antibody and only two clear

bands of heavy- and light-chain Ig. Strip material is also closely related to experimental results. Two NC membranes of different pore size and flow rate were compared and the HF135 NC membrane from EMD Millipore was more ideal. The concentrations of *E. coli* O157:H7 polyclonal antibody (T line) and rabbit antimouse IgG antibody (C line) were optimized and it was found that NC membrane was not sealed. A light red background may occur outside the T line and C line. Therefore, the composition and concentration of the sealing solution was also determined.

Specificity of ICA

After the strips are inserted into samples, colloidal gold-labeled antibodies combine with *E. coli* O157:H7-specific antigens. The antigen-antibody-colloidal gold particles move up by syphonage and combine with *E. coli* O157:H7 polyclonal antibodies in T line to form a microscopic red line. The remaining colloidal gold-labeled antibodies continue to rise and combine with rabbit antimouse IgG secondary antibodies in C line. If there is no *E. coli* O157:H7 in the samples, the red line will be formed only in the C line. The authors' results showed that *E. coli* O157:H7 of three strains were all positive and that other strains were negative. *E. coli* of ten different serotypes and non-*E. coli* of eleven different strains were enriched with IMPs and cultured with increased bacterium fluid. *E. coli* O157:H7 strains were positive, whereas other tested strains were negative. The results show that the immune chromatography dipsticks have very good specificity.

Sensitivity of ICA

E. coli O157:H7 fluid of concentration gradient 10^3–10^8 CFUs/mL was detected with ICA. The sensitivity of ICA was 10^5 CFUs/mL. This result was consistent with the sensitivity reported by Jung et al,[13] but the result was one order higher than that of the sensitivity of Fratamico and Bagi.[14] This may be caused by *E. coli* O157 polyclonal antibodies in this study.

Reproducibility and stability of ICA

The dipsticks from different batches, different storage temperatures, and different storage time were used to detect samples, and results indicated good reproducibility and a validity of at least 6 months.

Detection of IMPs combined with ICA

IMPs enriching *E. coli* O157:H7

IMPs of 10 μL, 30 μL, 50 μL, 70 μL, and 100 μL were each mixed with 1 mL *E. coli* O157:H7 of concentration gradient

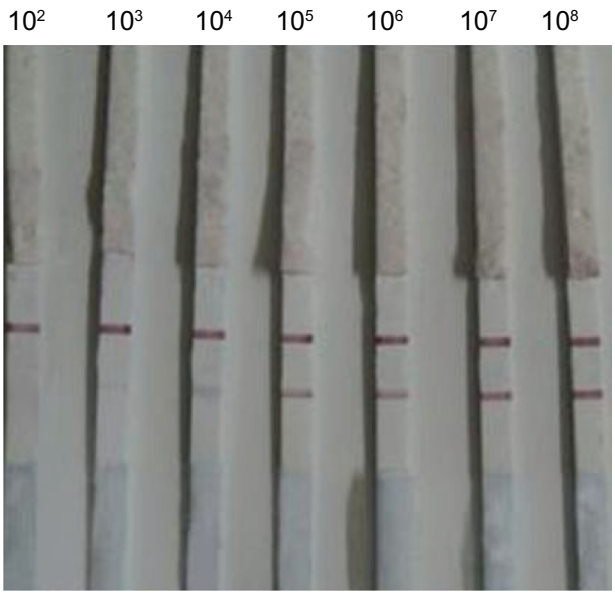

Figure 1 The sensitivity of *Escherichia coli* O157:H7 (1×10^8 to 1×10^2 CFUs/mL) with IMPs+ICA.
Abbreviations: CFUs, colony-forming units; ICA, immunochromatographic assay; IMPs, immunomagnetic nanoparticles.

10^2–10^8 CFUs/mL at room temperature for 10 minutes. Following magnetic separation and removal of supernatant, they were washed twice to obtain *E. coli* O157:H7. The *E. coli* O157:H7 underwent a colony count. When IMPs were more than 30 μL, the efficiency of IMPs gathering bacteria was basically maintained at about 10^5 CFUs/mL, which demonstrated that, by combining anti-*E. coli* O157:H7 antibody and IMPs close to saturation, the efficiency of IMPs gathering bacteria was not obviously increased. The efficiencies of IMPs gathering bacteria for 10^6, 10^5, and 10^4 *E. coli* O157:H7 were 6%, 22%, and 31%, respectively, demonstrating that the concentration of bacillus solution is lower, and the efficiency of IMPs gathering bacteria is higher. The optimal amount of IMPs for bacillus solution of different concentrations was 30 μL. If *E. coli* O157:H7 enrichment with IMPs is directed by ICA, there will be two problems. Firstly, IMPs

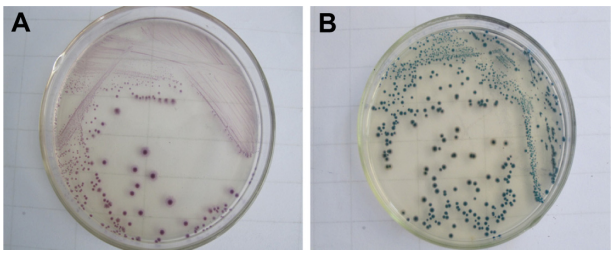

Figure 2 The specificity of IMP enrichment for *Escherichia coli* O157:H7. (**A**) *E. coli* O157:H7 will appear as aubergine colonies. (**B**) Non-*E. coli* O157:H7 will appear as blue-green colonies and no growth.

Table 1 Comparison of two detection methods for *Escherichia coli* O157:H7

Sample	Concentration (CFUs/mL)	N	ICA	Positive rate (%)	IMPs+ICA	Positive rate (%)
Milk	10^5	15	15	100	15	100
	10^4	15	0	0	15	100
	10^3	15	0	0	14	93.3
	0	5	0	0	0	0
Water	10^5	15	13	86.7	15	100
	10^4	15	0	0	15	100
	10^3	15	0	0	12	80.0
	0	5	0	0	0	0.0
Beef	10^5	15	12	80.0	15	100
	10^4	15	0	0	15	100
	10^3	15	0	0	13	86.7
	0	5	1	0	0	0

Abbreviations: CFUs, colony-forming units; ICA, immunochromatographic assay; IMPs, immunomagnetic nanoparticles.

can be delayed in the NC membrane due to their larger size, affecting ICA. Secondly, partial combining sites of antigens are occupied by a combination of IMPs with target bacteria, affecting the coloration produced by the combination of colloidal gold-labeled monoclonal antibodies with antigens. In this study, target bacteria were isolated with IMPs by heating to ensure the experiment was successfully carried out.

Sensitivity and specificity of IMPs combined with ICA

Dipsticks were used to detect the bacillus solutions mixed by 10^2–10^8 CFUs/mL *E. coli* O157:H7 and 30 μL of IMPs. The results indicated that 10^3 CFUs/mL and 10^4 CFUs/mL bacillus solutions were weakly positive, whereas 10^5–10^8 CFUs/mL bacillus solutions were positive and strongly positive. With the increase in the concentration of bacillus solution, the color above T line is increasingly clear (Figure 1). The data showed that the sensitivity of IMPs combined with ICA reached 10^3 CFUs/mL.

E. coli of ten different serotypes and non-*E. coli* of eleven different strains were enriched by IMPs. Following magnetic separation and removal of supernatant, the obtained bacteria were coated on *E. coli* O157:H7 chromogenic medium at 37°C for 24 hours. The results indicated that there was colony growth of *E. coli* O157:H7 (Figure 2, aubergine). There was no other *E. coli* or bacteria (Figure 2, blue-green color). It showed that IMPs failed to gather other strains and could only gather the target bacteria (Figure 2).

Table 2 The sensitivity and specificity of the two detection methods for *Escherichia coli* O157:H7

Group	N	ICA		IMPs+ICA	
		Sensitivity	Specificity	Sensitivity	Specificity
O157:H7	135	29.6%		95.5%	
Control	15		93.3%		100%

Abbreviations: ICA, immunochromatographic assay; IMPs, immunomagnetic nanoparticles.

Detection in food samples

When ICAs were used to detect *E. coli* O157:H7 (detection range $\geq 10^5$ CFUs/mL) in milk, purified water, and beef, the positive rate was 100% in milk, 86.7% in purified water, and 80% in beef, showing high sensitivity. However, with the outside test range ($\leq 10^5$ CFUs/mL), sensitivity was greatly reduced. Because IMPs enriched *E. coli* O157:H7, the positive rate of IMPs combined with ICA was still ≥80% when the concentration of bacillus solution was 10^3 CFUs/mL (Table 1). A total of 150 food samples (135 positive and 15 negative samples) were detected (Table 2). The sensitivity and specificity of ICA were 29.6% and 93.3%, respectively, and the sensitivity and specificity of IMPs combined with ICA were 95.5% and 100%, respectively.

Conclusion

A rapid and highly sensitive method for detection of *E. coli* O157:H7 was developed. When IMPs enriched for *E. coli* O157:H7 are combined with ICA they increase the sensitivity from $\geq 10^5$ CFUs/mL to $\geq 10^3$ CFUs/mL, and test results can be obtained within 1 hour. The method is simple, rapid, highly sensitive, specific, and does not require special equipment. The method potentially has a range of practical applications.

Acknowledgments

This work was financially supported by National 863 High Technology Project of China (No. 2003BA310A23), Technology Project of Shenzhen (No. 2004A110), and Technology Project of Guangdong (No. 2006B14701001).

Disclosure

The authors report no conflicts of interest in relation to this work.

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