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Full Length Research Paper

Development and evaluation of colloidal gold immunochromatographic strip for detection of *Escherichia coli* O157

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***Escherichia coli* O157:H7 is a serious and common human pathogen that can cause diarrhoea, haemorrhagic colitis, and haemolytic uraemic syndrome (HUS). In this study, the synthesis and identification of colloidal gold particles and antibody-colloidal gold conjugates probe specific to *E. coli* O157:H7 were performed, and the preparation of colloidal gold immunochromatographic strip based the biotin-streptavidin system was developed for detection of *E. coli* O157:H7.Monodispersional nanogold colloid was synthesized and preparation of nanogold-labeled polyclonal antibody probe to *E. coli* O157:H7 by citrate method. Combination of antibody with nanogold particles was also characterized by UV-visible (UV-vis) light absorption spectra and transmission electron microscopy (TEM). Furthermore, nanogold-labeled probe was used to develop an immunochromatographic (IC) strip for *E. coli* O157:H7 analysis. With this method, analysis could be completed in less than 10 min. Examination of the 65 known strains (36 *E. coli* O157 strains and 29 serotypes other than *E. coli*) showed 98.5% specifity and 100% sensitivity, only yield a false-positive reaction with *Salmonella choleraesuis*. The sensitivity of the IC strip was tested using 10-fold dilution *E. coli* O157 in foods, could be detected at a minimum of 2.3 × 10^3 CFU/ml without enrichment and 2.3 CFU/ml after enrichment. Application of IC strip test were performed on 265 water samples, 340 beef samples, 208 milk samples and 120 cake samples after enrichment, the specificity of the strip was 99.2, 97.9, 94.6 and 94.9%, respectively. The sensitivity of the strip was 100% agreement with tradition culture method. The established method is very useful for monitoring *E. coli* O157 containment in food samples.**

Key words: *Escherichia coli* O157, colloidal gold immunochromatographic assay, rapid test.

INTRODUCTION

Escherichia coli O157:H7, member of *Enterohemorrhagic E. coli* (EHEC), is a bacterium that causes diarrhea that is often bloody. The diarrhea can be accompanied by abdominal cramps. Fever may be absent or mild. Symptoms usually occur within 2 - 3 days following exposure, but may occur as soon as 1 day following exposure or up to one week following exposure (Reinstein et al., 2007; Erdogan et al., 2008). Healthy adults can typically recover completely from *E. coli* O157:H7 exposure within a week. However, some people, especially young children and the elderly, can develop Hemolytic Uremic Syndrome (HUS) as a result of exposure to *E. coli* O157:H7, a condition that can lead to serious kidney damage and even death (Sanchez et al., 2008, Baudouin et al., 2008).

The contamination of food with *E. coli* O157:H7 is of increasing concern internationally. However, in a traditional method, the contaminated food sample or feces is allowed to grow in an enriched media for 6 - 18 h a portion of the broth then plated on agar media and analysed by biochemical tests and serological reactions which are time-consuming, laborious, and inefficient. Other methods such as the immunomagnetic beads

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coated with antibodies of *E. coli* O157:H7(Crawford et al., 2000; Shelton and Karns, 2001; Tu et al., 2003; Gehring and Tu, 2005a) or the more established polymerase chain reaction (PCR) (Elizaquivel and Aznar, 2008; Gooding and Choudary, 1998; Takeshi et al., 1997), loop-mediated amplification (LAMP) (Hara-Kudo et al., 2008; Xihong Zhao, 2010), where the relevant genes are amplified, require trained personnel and clean samples that may need several hours of incubation to obtain detectable amounts of the target. Although enzyme immunoassay has been used widely in many laboratories (Gehring et al., 2006; Chapman et al., 1997; Fratamico and Strobaugh, 1998; Feldsine et al., 2002; Bai et al., 2007), this method still requires various equipments and trained personnel. Thus, a simple and inexpensive immunochromatographic strip could have diagnostic applications without the requirement of complicated equipment. Jung, et al (Jung et al., 2005) developed the colloidal immunochromatographic strip for detection of *E. coli* O157 in enriched samples, reported that the minimum limit was 1.8×10^5 CFU/ml without enrichment and 1.8 CFU/ml after enrichment. However, there were some problems with the development of immunoassay methods, such as the limit of detection and false positive (Gehring and Tu, 2005b).

Avidin and streptavidin are widely used in biotin - (strept) avidin system technology, which is based on their tight biotin-binding capability (Laitinen et al., 2007). These biotin-(strept) avidin based methods enable both signal amplification and a reduction in background activity (Hartley, 1996) .The immunoassay method based biotin-streptavidin signal amplification system was developed suitable analytical techniques to be used in many fields detection. (Paffard et al., 1997; Roth and Jeltsch, 2000; Matalka et al., 2001; Rambozzi et al., 2004; Yu et al., 2007). But there were no articles about the colloidal gold immunochromatographic strip based biotin-streptavidin system for detection *E. coli* O157.

We have developed a rapid two-step procedure, coated streptavidin (SA) in Test line of nitrocellulose menbrane targeting moiety, colloidal gold labeled the anti- *E. coli* O157 monoclonal antibodies (MAbs) and biotinylated anti- *E. coli* O157 polyclonal antibody were impregnated in the conjugated pad and sample pad, respectively. The advantage of SA coating in the Test line, compared to the use of directly MAbs, was a reduction in background levels and amplification sign due to rapid clearance of biotinylated antibody and capture four times molecular biotin. Also, we presented details of our investigation into the formation and characterization of an antibody–colloidal gold probe (conjugate) specific to *E. coli* O157:H7 and its use in developing a rapid *E. coli* O157:H7 diagnostic method. The formation of antibody and colloidal gold conjugate was accomplished at optimal condition. The processes of formation of colloidal gold and conjugate were monitored by UV-visible (UV-vis) light measurements, whereas transmission electron microscopy (TEM) images were used to characterize the particle size of the conjugates. Furthermore, nanogold probe was applied in developing an immunochroma-tographic assay based the biotin-streptavidin system for *E. coli* O157:H7. We also estimated the specificity and sensitivity of the immunochromatographic strip with pure cultured bacteria and various enriched food samples.

MATERIALS AND METHODS

Bacteria strains and reagents

E. coli O157 strains, *E. coli* Non-O157 strains, Non-*E. coli* strains were obtained from the Zhongshan Supervision Testing Institute of Quality and Metrology at Zhongshan city, Guangdong province, China. All cultures were maintained on Tryptic Soy Agar (Remel, Lenexa, KS) slants or plates. Food samples (water, beef, cake and milk) were bought from an open market near the campus of South China University of Technology in the city of Guangzhou, China. Cultures for the assays were grown on tryptic soy broth for 18 h at 37℃ with aeration and serially diluted 10 fold in sterile 10 mmol/l phosphate buffered saline (PBS) at pH 7.4. The number of colony forming units (CFU), for each dilution was confirmed by plating 100 µl of diluted sample onto tryptic soy agar plates and incubated at 37℃ for 24 h. The colonies formed on the plates were then counted.

Nitrocellulose membrane, glass fiber and absorption pad were obtained from sartorius stedim (Germany). Chloroauric acid ($HAuCl_4\cdot3H_2O$) and sodium citrate were obtained from the chemicals and reagents of Shanghai and used without further purification. EZ-link sulfo-NHS-LC-Biotin and streptavidin (SA) were obtained from Pierce Chemical Co. (Rockford, IL, USA). Deionized and distilled water was used in this experiment. All other chemicals used in the present study were either analytical pure or with highest quality.

Preparation of biotinylated antibody

Preparation of biotinylated rabbit anti- *E. coli* O157 polyclonal antibody was carried out using EZ-link sulfo-NHSLC-Biotin according to the manufacturer's instructions for biotinylation of proteins.

Synthesis and characterization of colloidal gold

Colloidal gold particles with a mean diameter of 40 nm were prepared according to the method by Xu et al (Huo et al., 2006). Briefly, an aqueous solution of chloroauric acid (30 ml of 0.01% (*W*/*V*) $HAuCl_4\cdot3H_2O$) was heated to boiling point, followed by 300 µl of 1.0% (*W*/*V*) sodium citrate solution added into it (Ji et al., 2007). The reaction solution was stirred simultaneously and gently boiling it for 15 min until the color of the solution turned from straw yellow into black and eventually red. The obtained colloidal gold solution was used for conjugation with monoclonal antibody (MAb) to *E. coli* O157:H7.

Minimum amount of MAb for stabilizing colloidal gold particles

A murine monoclonal antibody against *E. coli* O157:H7 lipopolysaccharide was used in this research .For the conjugation of colloidal gold, a minimum amount of MAb concentration was needed for stabilizing colloidal gold particles. Colloid gold suspension was adjusted to the same pH (ranges about 7 - 8) with 0.01 mol/L Na_2CO_3 solution firstly and pipetted into a series of tubes

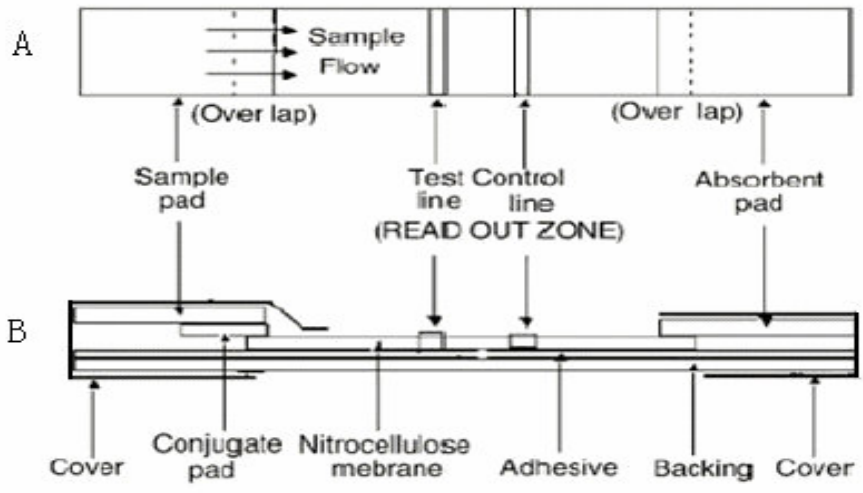

Figure 1. Schematic representation of the lateral flow immunochromatographic test strip. (A) Top view; (B) cross-section.

(tube/0.95 ml). Then, monoclonal antibody solution (0.01 - 0.07 mg/ml, 0.95 ml) was added to the above each colloidal gold solution. After that, each tube received 0.1ml of 10% NaCl and was shaken for observation of color change after 2 h. In the experiment, 0.03 mg/ml of MAb was confirmed to be the minimum amount for stabilizing colloidal gold solution. Therefore, 2.5 ml MAb (0.03 mg/ml prepared in pH 7.4, PBS 0.01 mol/L) was added drop-wise to 2.5 ml of colloidal gold solution. The solution was stored for 2 h at 4℃ and then, was centrifuged to remove unconjugated MAb. The centrifugation was undertaken at 14,000 r/min at 4℃ followed by the pellet redispersed in total 5 ml of pH 7.4 PBS and stored at 4℃ for further experiments.

Characterization of conjugates

UV-vis spectroscopy studies

The formation of antibody - colloidal gold conjugates was monitored by UV-vis spectroscopy (200 - 700 nm) using a double-beam spectrophotometer (model 1.70, GBC) operated at 1 nm. The gold solutions were monitored immediately after addition of the antibody and after centrifugation and resuspension of the conjugates in appropriate buffers.

Transmission electron microscopy measurements

TEM measurements were performed on a Hitachi H-7000 microscope operating at an acceleration voltage of 100 kv at a magnification of 100,000. The sample was prepared by placing a drop of colloidal gold and centrifuged and resuspended antibody - colloidal gold solution on a carbon-coated TEM copper grid. The film was allowed to dry for 5 min, and the excess solution was removed using a blotting paper.

Samples preparation

Samples of ground beef were prepared by mixing 5 g of ground beef with 45 ml of PBS spiked with *E. coli* O157:H7 at concentrations ranging from 10^1 - 10^7 CFU/ml. The samples were homogenized by stomaching for 1 min so that the heavier particulates could settle before pipetting the clear supernatant into a vial for analysis. Ground beef sample extract in PBS with no pathogen were used as negative control. A similar method was adopted for cake (Waswa et al., 2007).

Pasteurized skimmed milk (50 ml) was inoculated with *E. coli* O157:H7 at about 10^7 CFU/ml. The inoculated sample was then taken through a 10-fold serial dilution in uninoculated pasteurized milk to obtain samples in the concentration range between 10^1 and 10^6 CFU/ml. Uninoculated samples were used as negative control . A similar method was adopted for water (Huang et al., 2007). Each 80 µl sample applied on test strips.

Preparation of immunochromatographic test strip

Immunochromatographic strip was constructed as a method by Huo et al., 2006 and Lakner et al., 1998 Colloidal gold-labeled antibody conjugate and biotinylated antibody were jetted onto glass fiber (conjugate pad) and non woven (sample pad) , respectively, then dried at 50℃ for 2 h. Goat anti-mouse antibody (1.0 mg/ml) was dispensed onto a nitrocellulose membrane on the upper line for control with a volume of 1 µl per 1 mm line, and SA (1.0 mg/ml) in PBS was jetted into the lower part for test line; the dispensed volume was also of 1 µl per 1 mm line. The remaining active sites on the membrane were blocked by incubation with 5% BSA in PBS (1 ml/cm membrane) for 30 min at 37℃.The membrane was washed once with PBS and again with distilled water and then, dried at 37℃. Subsequently, the absorption pad, the nitrocellulose membrane, the released antibody-gold conjugation pad, and the sample application pad were assembled into a sheet of plastic backing orderly (Figure 1) and cut into individual strips (2.0 mm/strip) with a pair of small medicine scissors. The total assay time was less than 10 min.

Sensitivity and specificity

The sensitivities and specificities of the tests were determined for the evaluation of the utility of the tests. The following formulas were used to calculate test indices (McClure, 1990): sensitivity = [TP/(TP + FN)] × 100 and specificity = [TN/(TN + FP)] × 100 (where TP= number of true positives, TN = number of true negatives, FN = number of false negatives, and FP = number of false positives).

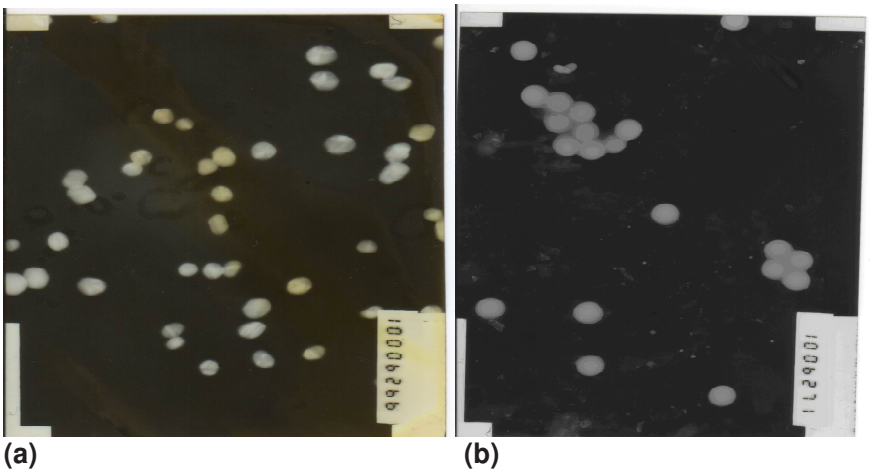

Figure 2. TEM image of particles (×100,000) (a) Colloidal gold particles; (b) antibody - colloidal gold conjugates particles.

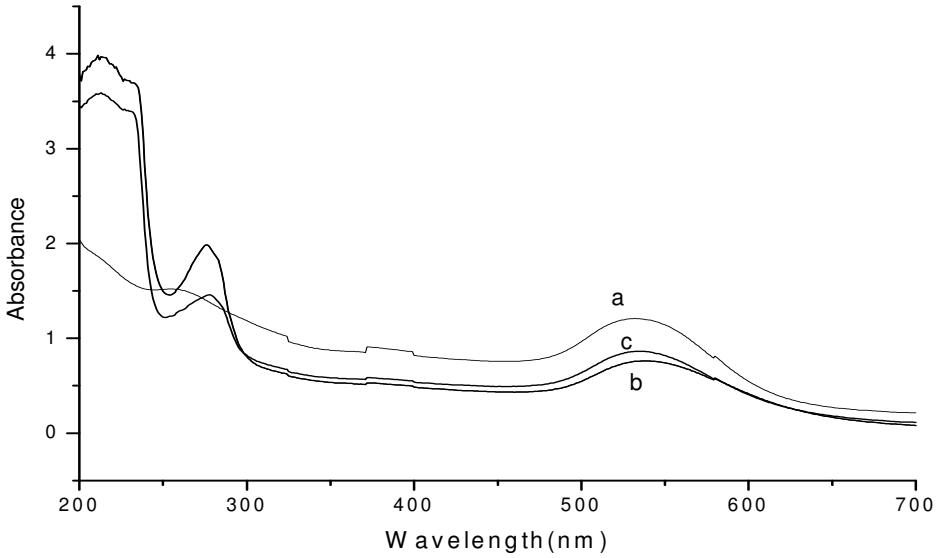

Figure 3. UV/Vis spectra of colloidal gold and antibody-gold conjugate. Curve a: colloidal gold solution; curve b: conjugate immediately after addition of antibody to gold solution; curve c: conjugate in buffer after centrifugation and resuspension

RESULTS AND DISCUSSION

Characterization of the colloidal gold particles

Nanogold colloid particles were synthesized by chemical condensation using the reduction of chloroauric acid. Chloroauric acid was reduced to gold atoms by the sodium citrate, and many of gold atoms accumulate into nanogold particles. The TEM image showed well-dispersed colloidal gold particles (Figure 2a).The average diameter of the colloidal gold obtained was about 40.0 nm. TEM images indicated that the gold colloid were almost of the same diameter, which provided the basis for probe preparation of immuochromatography test strip.

Characterization of antibody–gold conjugates

UV-vis spectra

Figure 3 showed the UV-vis spectra of the colloidal gold and antibody-colloidal gold conjugates. A peak at ~532 nm in curve a was a result of the surface plasmon resonance of colloidal gold particles. After addition of the antibody, the surface plasmon band shifted a litter into

the red direction which caused by the interaction of the antibody with colloidal gold particles. A peak at ~280 nm (curve b) in the conjugate system arose due to the tryptophan and tyrosine residues in the protein of monoclonal antibody (Gole et al., 2002). After centrifugation and resuspension of the conjugate (curve c), the intensity of the surface resonance band and protein absorption at 280 nm decreased because of reduced colloidal gold concentration and removal of the excess unconjugated monoclonal antibody molecules in solution.

TEM images

Figure 2b showed the TEM images of the antibody–colloidal gold conjugates formed on a carbon-coated copper grid. It was observed that the gold particles were still monodisperse and ordered as the colloidal gold. The mean size of the particles was 42 nm, which was propitious for strong signal generation in immuochromatography test strip.

Immunochromatographic test

The lateral flow assay result was determined visually by the degree of intensity of the gold color of the test line and the control line on which the SA and goat anti-mouse IgG were separately immobilized, and the result was immediately observable. If *E. coli* O157 is present in the sample, it binds to the biotinylated antibody in the sample pad first to form biotinylated antibody-antigen complex. Then the complex reacts with colloidal gold-labeled anti-*E. coli* O157 Mab to form biotinylated-colloidal gold complex (biotinylated antibody-antigen-colloidal gold body complex), then migrated by capillary action through the membrane on the detection zone. A red band appeared within 5 min as biotinylated-colloidal gold complex capturing by SA as the high-affinity biotin-streptavidin interaction in test line. However, the excess colloidal gold conjugates moved farther until they reacted with goat anti-mouse IgG, showed another red line on the upper portion of the NC membrane. Therefore, two red lines on the IC strip indicated the presence of *E. coli* O157. In contrast, negative samples resulted in only one red line between colloidal gold conjugates and anti-mouse IgG at the upper portion of the NC membrane. If no line forms, the test is invalid.

Strains

Table 1a showed the results of the immunochromatographic test pure-cultured bacteria. All *E. coli* O157 strains reacted strongly with immunochromatographic strip, regardless of H serotype. While the other strains, representing 10 *E. coli* Non-O157 serotype, did not react with it. However, one (*Salmonella choleraesuis*) of the 19 tests strains of non- *E. coli* yielded false-positive reactions. It was reported that some of the non-*E. coli* strains, for example *Salmonella, Citrobacter freundii* and *Escherichia hermannii*, shared common antigens with *E. coli* O157 (Borczyk et al., 1990; Bettelheim, 1993). However, the disadvantage could be conquered by following selective plating media; *Salmonella* was inhibited the growth on cefixime-tellurite sorbitol MacConkey agar as well as fermented sorbitol (Fratamico and Bagi, 2007; Jung, 1999).

Table 2b showed the summary of the *E. coli* O157 immuochromatography strip test of pure-cultured bacteria. All *E. coli* O157 strains were correctly identified, so the sensitivity of the immunochromatographic test was 100%. One yielded a false-positive reaction, so the specificities of the test was 98.5%. Therefore, the false-negative rate was 0% (0/28) and false-positive rate was 1.5% (1/65).

Detection of *E. coli* O157 in foods

In the present study, the sensitivity of the IC strip was evaluated using 10-fold diluted *E. coli* O157:H7 with a range of 2.3×10^6 to 2.3 CFU/ml. *E. coli* O157 could be detected at a minimum of 2.3 CFU/ml after enrichment (Figure 4) and 2.3×10^3 CFU/ml without enrichment. The results showed that the sensitivity of the IC strip enhanced highly with sample after enrichment. Because of small numbles of *E.coli* O157:H7 in samples and food matrix reasons, we suggest that combining the enrichment procedures and IC test step.

When we applied the IC strips using food samples in the field, 8 of 265 water samples, 5 of 340 beef samples, 10 of 208 milk samples, and 5 of 120 cake showed positive signals. In contrast, *E. coli* O157:H7 were isolated from 6 (2.3%) of 265 water samples, 5 (1.5%) of 340 beef samples, 5 (2.4%) of 208 milk samples, and 3 (2.5%) of 120 cake samples (Table 2). We found that results obtained with the IC strip exhibited 100% agreement with those of traditional cultured methods after selective enrichment, since *E. coli* O157:H7 was then isolated from all the samples with positive strip test results.

Prior to constructing the one-step sandwish immunochromatographic assay strip by using nanogold–antibody conjugate probe, we employed two-step immunochromatographic assay. In order to enhance the sensitivity of detection, we selected the biotin-streptavidin amplification system. The first step was that biotinylated antibody and colloidal gold labeled Mab captured the *E. coli* O157:H7 from samples, then formed the complex. The second step was that the complex was captured by SA for high-affinity biotin-streptavidin interaction. Results showed that the sensitivity was near 100 times higher than one-step sandwish immunochromatographic assay strip by Jung et al. (2005) with pre-cultured strains in food.

Table 1a. Specificity of the *E. coli* O157 immuochromatographic strip tested with different pure-cultured bacteria.

Microorganism	No. of strains tested	No. of positive tested	No. of negative tested
E. coli O157		c	
E. coli O157:H7	32	32	0
E. coli O157:H19	2	2	0
E. coli O157:H43	1	1	0
E. coli O157:H45	1	1	0
E. coli Non-O157			
E. coli O3	3	0	3
E. coli O26	2	0	2
E. coli O38	1	0	1
E. coli O111	3	0	3
E. coli O145	1	0	1
Non- *E. coli*			
Salmonella typhimurium	1	0	1
Salmonella choleraesuis	2	1	1
Salmonella paratyphi	1	0	1
Shigella flexner	1	0	1
β-Hemolytic streptococcus	1	0	1
Enterococcus faecalis	2	0	2
Vibrio parahaemolyticus	3	0	3
Staphyloccs epidermidis	1	0	1
Staphylococcus albus	2	0	2
Streptococcus faecalis	1	0	1
Staphylicoccus aureus	2	0	2
Pseudomonas aeruginosa	2	0	2

Table 1b. Summary of the *E. coli* O157 immuochromatographic strip test of different bacteria.

Microorganism	**No. of strains tested**			**Specificity**	**Sensitivity**
	Total	**Positive**	**Negative**	**(%)**	**(%)**
E. coli O157	36	36	0	98.5	100
E.coli Non-O157	10	0	10		
Non- *E. coli*	19	1	18		

Table 2. Comparison detection *E. coli* O157:H7 of immunochromatographic strip test with traditional isolation

***E. coli* O157:H7 isolation**	**No. of positive/ negative samples by immunochromatographic strip test**			
	Water (n = 265)	**Beef (n = 340)**	**Milk (n = 208)**	**Cake (n = 120)**
Positive	6/0	5/0	5/0	3/0
Negative	2/247	7/329	11/192	6/111
Sensitivity	100.0	100.0	100.0	100.0
Specificity	99.2	97.9	94.6	94.9

Conclusion

Monodisperse nanogold colloid was prepared with a reduction of chloroauric acid by sodium citrate. An antibody probe to detect *E. coli* O157 was prepared by addition of the monoclonal antibody to the colloidal gold

Figure 4. Sensitivity of *E. coli* O157 immunochromatographic strip with enrichment. *E. coli* O157:H7 was spiked and then 10-fold diluted from 2.3 × 10^6 CFU/ml (strip a) to 2.3 CFU/ml (strip g). Strip h was a negative control. The strip could detect 2.3 × 10^3CFU/ml before enrichment; it could detect 2.3 CFU/ml (strip g) after enrichment. Strip i was an unvalid control.

solution to form conjugates. Optimal conditions for conjugation between colloidal gold and antibody were also ascertained. UV-visible light absorption spectra and transmission electron microscopy images showed that the nanogold and nanoprobe gold particles were well defined and regularly shaped.

The proposed method has been used for the determination of *E. coli* O157 in food samples with satisfactory results. Compared with the traditionally colloidal gold immunochromatographic detection, this method based the biotin-streptavidin system showed greatly heightened sensitivity. The minimum limit of detection improved by near two orders of magnitude, which was very suitable for the conditions with extremely low concentration of analysis or very small volumes of sample on site. The method could be obtained within 10 min and that all needed reagents were included in the strip, also referenced in the development of immunochromatographic assay for the detection of other food-borne pathogen.

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