Note

Rapid Immunochromatographic Assay for *Escherichia coli* O157:H7 in Bovine Milk Using IgY Labeled by Fe₃O₄/Au Composite Nanoparticles

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A rapid pretreatment-free immunochromatographic assay (ICA) was developed for the screen of *Escherichia coli* O157:H7 (*E. coli* O157:H7) in bovine milk. Anti-*E. coli* O157:H7 immunoglobins of yolk (IgY) was conjugated with a core/shell-structured super-paramagnetic Fe₃O₄/Au composite nanoparticle (GoldMag) and showed a high coupling ratio of 8 µg IgY mL⁻¹ nanoparticles at pH 8.0. The conditions of ICA strips using this IgY conjugate were optimized and could be used to measure spiked *E. coli* O157:H7 samples with a detection limit of 10³ CFU mL⁻¹ obtained by visual detection. Additionally, the magnetic signal intensities of the assay can quantitatively measure *E. coli* O157:H7 concentration in the range of 10² – 10⁵ CFU mL⁻¹ both in phosphate buffer solution (pH 7.4) and pretreatment-free milk samples with a detection limit of 10² CFU mL⁻¹.

Keywords: *Escherichia coli* O157:H7, magnetic immunochromatographic assay, Immunoglobins of yolk (IgY), Rapid screening, quantitative measurement

Introduction

Escherichia coli O157:H7 (*E. coli* O157:H7) is a widespread food-borne pathogen and capable of producing toxin and causing severe gastrointestinal disease in humans. It may lead to hemolytic uremic syndrome (HUS), and even to death, especially in children < 5 ages occasionally. *E. coli* O157:H7 can be transmitted through foods and water, which is estimated to cause 73,000 illnesses occurring each year (Mead *et al.* 1999). Dairy cattle are considered reservoirs of STEC (Enterotoxigenic *E. coli*) including *E. coli* O157:H7 and can impose a significant health risk to humans (Hussein and Sakuma 2005). The transmission to humans is

associated mostly with consumption of contaminated food, unpasteurized milk and ready-to-eat products (Liptakova *et al.* 2004). The infective dose of *E. coli* O157:H7 is reported as low as 50 – 100 cells (Strachan *et al.* 2001). In addition, its extended survival in bovine feces emphasizes that environmental contamination may pose a serious risk to raw milk and ground beef. Milk-borne outbreaks of *E. coli* O157:H7 have been reported worldwide (Keene *et al.* 1997; Gaulin *et al.* 2012). In a review conducted by Baylis that numerous cases of food poisoning by *E. coli* O157:H7 due to unpasteurized milk products consumption were reported (Baylis, 2009). Therefore, prompt, careful

List of abbreviations

Escherichia coli O157:H7 - *E. coli* O157:H7; IgY-immunoglobins of Yolk;

ICA-immunochromatographic assay; GM - Goldmag, A core/shell-structured super-paramagnetic Fe₃O₄/Au composite nanoparticles;

Magnetic Assay Reader - MAR; Relative magnetic units - RMUs; Magnetic signal -MS; ELISA Index -EI; Nitrocellulose -NC

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monitoring and control of *E. coli* O157:H7 is extremely important to food safety evaluation.

Various methods have been developed to determine *E. coli* O157:H7 contamination in the food matrix. Conventional techniques used for detection of the *E. coli* O157:H7 in cases of food poisoning include bacteriological methods (Gehring *et al.* 2012), polymerase chain reaction (PCR) (Wang *et al.* 2014), surface plasmon resonance (Wang *et al.* 2013) (SPR), enzyme-linked immunosorbent assays (ELISA) (Cavauiuolo *et al.* 2013), flow cytometry (Yankey *et al.* 2012) and so on. Culture-based methods are labor intensive, time consuming, and not sensitive. PCR yields fast results but it is prone to be affected by inhibitors in a food matrix. The other methods may need complicated preconcentration, time-consuming steps and high-cost instruments. Those rent themselves poorly to out-of-laboratory scenarios and on-spot detection.

Lateral-flow immunochromatographic assay (ICA) has been widely applied in the field of food safety supervision (Berlina *et al.* 2013; Byzova *et al.* 2014). This technique utilizes antigen-antibody assay and provides rapid detection of analytes. The results judging by naked eye ensure the convenience of bioassays on-site. Colloidal gold was commonly used as a marker in ICA, mainly because of the nano-diameter and red color. A core/shell-structured super-paramagnetic Fe₃O₄/Au composite nanoparticle (GoldMag) has been introduced into immunochemistry as a marker for a visible color reaction to develop an ICA (Yang *et al.* 2013), which can take advantage of magnetic intensity except the red color over the colloidal gold. The sensitive and specific of ICA strip was determined by not only labeling marker used but also the antibodies. Immunoglobins of Yolk (IgY) is a kind of chicken antibodies extracted from egg yolks, which have been used as an alternative to mammalian IgG for serological tests (Cova 2005).

We describe the development of a sandwich ICA for *E. coli* O157:H7 screening in bovine milk, using IgG as the capture antibodies and modified IgY as the detecting antibody. This method has some distinct advantages over traditional immunoassays- ease of procedure, rapid operation, low-cost and immediate results.

Material and Methods

Bacterial strains The bacteria strains used in this study were listed in Table 1. *E. coli* O157:H7 ATCC 43895 was taken as the reference strain. It cannot ferment sorbitol. All bacteria were cultured, harvested and inactive in formaldehyde (0.3 – 0.4%) to obtain the suspensions (10⁹CFU mL⁻¹) for use.

Preparation of GoldMag and IgY conjugate and immunochromatographic test strips Polyclonal anti-*E. coli* O157:H7 IgY was produced as previously reported (Sunwoo *et al.* 2006). Purified IgY was used as a detection antibodies and conjugated to GoldMag (GM, Shaanxi Lifegen Co., Ltd, China). To examine a proper amount of antibodies and pH values for stabilizing GM nanoparticles, IgY-GM titration were performed

Table 1. Bacteria strains used in this study.

Bacteria strains		Source
<i>Escherichia coli</i> O157:H7	ATCC43895	CN-CDC ^a
	ATCC43889	CN-CDC
	ATCC43888	CN-CDC
	CMCC44828	CMCC ^b
	NCTC12900	NCTC ^c
	99X131	JS-CDC ^d
	99X132	JS-CDC
<i>STEC</i> O26:H11	ATCC BBA-2196	CN-CDC
<i>STEC</i> O111	ATCC BBA-2440	CN-CDC
<i>STEC</i> O103:H2	ATCC BBA-2210	CN-CDC
<i>STEC</i> O145	ATCC BBA-2206	CN-CDC
<i>non-pathogen E. coli</i> strains	BL21 DE3	Lab-preserved
	BL21RIPL	Lab-preserved
	JM109	Lab-preserved
	CMCC 44102	Lab-preserved
	ATCC 25922	Lab-preserved
	CMCC44103	Lab-preserved
<i>vulgaris E. coli</i>	CMCC 44102	Lab-preserved
<i>Enteroinvasive E. coli</i>	CMCC 44350	Lab-preserved
<i>Staphylococcus aureus</i> strains	CMCC26001	Lab-preserved
	CMCC26003	Lab-preserved
<i>S. typhimurium</i>	ATCC 13311	Lab-preserved
	<i>S. urbana</i>	Lab-preserved

^aCN-CDC: Chinese Center for Disease Control and Prevention

^bCMCC: China Medical Culture Collection, China

^cNCTC: National Collection of Type Cultures, China

^dJS-CDC: Jiang Su Province Center for Disease Control and Prevention, China

following a modified colloidal gold conjugation protocol (Rudolf *et al.* 2009). The absorption difference ($\Delta\text{Abs} = \text{Abs}_{550} - \text{Abs}_{600}$) used to estimate the optimum conjugation condition, where the difference reaches a constant level.

The ICA strip was assembled according to Byzova *et al.* (Byzova *et al.* 2014). 25 μL conjugate solution was sprayed on the conjugate pad, which was prepared by diluting with 0.02 M PBS (pH 8.0, 0.2% Tween-20, 1% PEG-2000, 1%BSA, 8% (w/v) sucrose) at ratios of 1:1. Pure cellulose fiber of 100% was used as the absorbent pad. The test line was coated by serially diluted IgG anti- *E. coli* O157:H7 (T line, Beijing Bioss Co., China), 0.3 mg mL⁻¹, 0.4 mg mL⁻¹, 0.5 mg mL⁻¹ and 0.6 mg mL⁻¹ with the volume of 10 μL . The control line was coated with 10 μL of rabbit anti-chicken IgG (C line, Beijing Bioss Co., China, 0.5 mg mL⁻¹). Positive solutions containing *E. coli* O157:H7 (1 $\times$ 10⁵CFU mL⁻¹) were tested to determine the optimal concentrations of coating antibody.

200 μL *E. coli* O157:H7 simulated sample was tested by the strips and the different intensity of the red color can be observed by naked eye. The magnetic signal (MS) of the cartridge mentioned above was detected by Magnetic Assay Reader (MAR, Assay Development System, Magna-biosciences, LLC, USA). Data were

Fig. 1. Titration curve of IgY-GM conjugate, Absorbance difference between 550 nm and 600 nm. (A) Titration curve for the optimal concentration of IgY coupled with GM. (B) Titration curve of IgY coupled with GM at different pH.

reported as relative magnetic units (RMUs) which are in direct proportion to the amount of magnetic complexes formed in the T line (IgG-antigen-IgY-GM complexes) or C line (2nd IgG-IgY-GM complexes). All experiments were performed in Triplicate, and the average of the Triplicates was used in the analysis.

Sensitivity and specificity test *E. coli* O157:H7 was diluted from 1×10^1 to 1×10^6 CFU mL⁻¹ to evaluate the sensitivity of the strips. And Cow's milk with a fat content of 1.5% and 3.5% was purchased in retail stores. Formaldehyde inactive *E. coli* O157:H7 was added to the milk to generate the simulated milk samples in the concentration range of 1×10^1 to 1×10^6 CFU mL⁻¹. 200 μ L of each particular dilution was used to assess the sensitivity and PBS (0.02 M, pH 8.0) was used as the blank control.

The formaldehyde (0.3 – 0.4%) inactive bacteria solutions, including other kinds of *E. coli* O157:H7 strains, Non-pathogenic laboratory *E. coli*, *Salmonella* and *Staphylococcus aureus* with the same concentration of 10^5 CFU mL⁻¹ in milk samples were prepared to evaluate the specificity of the ICA strip.

All ICA strips were stored at 4°C for 12 weeks to evaluate the stability during storage. 1×10^5 CFU mL⁻¹ of *E. coli* O157:H7 and *E. coli* in milk was used as samples, with PBS (0.02 M, pH 8.0) as the blank control.

Results and Discussion

Optimal conditions for conjugation Different concentrations from 0 to 20 μg antibody mL⁻¹ GM solutions were prepared to estimate the optimal conditions for IgY-GM conjugate coupling. The optimal coupling ratio was determined at pH 8.0. As shown in Fig. 1A, the optimal IgY concentration for labeling was 8 μg IgY mL⁻¹ GM particles, where the saturation curve reached a plateau suggesting the colloidal particles stabilized. The amount of IgY used for GM conjugation was usually in excess by 30%, that is, 10.5 μg IgY mL⁻¹ GM particles.

The pH-value has a high impact on the charge state of IgY and GM particles. Considering that the pI of IgY is not a clearly defined point but rather an area of about two pH units, the GM solution was adjusted to 4 different pH values between 6.8 and 8.5 with 0.1 M K₂CO₃ to find the ideal conditions for antibody coupling. Fig. 1B showed that the saturation curve reached a constant level at pH 6.8, 7.5, 8.0, yet this saturation cannot be reached at pH 8.5. It might be that the pI of IgY are lower than that of IgG and then the repellants between antibody and GM particles are too high to cover particles with enough antibodies to reach a stable state at pH 8.5. However, more alkaline pH values are benefit for antibody binding to GM particles via their Fc regions (Dávalos-Pantoja *et al.*, 2001), the optimal pH was determined as pH 8.0.

Optimization of the ICA conditions In terms of the coating of IgG anti-*E. coli* O157:H7 at T line, the color intensity deepened gradually with the increase of concentration, and maintained when the IgG concentration reached 0.5 mg mL⁻¹. The appropriate amount applied to NC membrane test line was 10 μ L at concentration of 0.5 mg mL⁻¹.

For sandwich lateral flow test, the sample plays an important role of providing adequate quantities of antigen to link the two antibodies. More sample volume provides more *E. coli* O157:H7 at the same concentration. Hence the sample volume was tested. No significant growth of red color intensity was observed when the volume reaching 200 μ L. The reason might be that it is hard to complete the reaction between antigen and antibody in a solid-phase sandwich form in short time. The excess *E. coli* O157:H7-IgY-GM complex may just pass through the test line without capturing by IgG anti-*E. coli* O157:H7. As a result, 200 μ L was determined as the optimal sample volume for use.

Performance and sensitivity of ICA test strip The experiments of the ICA using the optimized test strip and *E. coli* O157:H7 standard solutions range from 1×10^1 – 10^6 CFU mL⁻¹ were

Fig. 2. Representative results of the lateral flow ICA for standard solutions of *E. coli* O157:H7.

performed in triplicate. The representative results are showed in Fig. 2. It can be seen that a negative control without *E. coli* O157:H7 gave only a clear red color at the control line and the same intensity of red color appeared on control lines in different strips, indicating validity of the assay. *E. coli* O157:H7 concentrations at 10^1 caused no color and 10^2 CFU mL⁻¹ caused weak color development at the test line. While, with the concentration of *E. coli* O157:H7 increased, the intensity of red color on test lines was appeared and increased. Furthermore, the intensity of red color on test line at 10^3 CFU mL⁻¹ of *E. coli* O157:H7 caused a distinguishable difference compared to the negative control. Therefore, the visual detection limit for *E. coli* O157:H7 was considered to be 10^3 CFU mL⁻¹. In addition, we also evaluated the effect of a food sample matrix on the ICA strips. A series of test strips was applied to characterize the efficiency of the *E. coli* O157:H7 detection in milk matrices. Compared to *E. coli* O157:H7 detection in a buffer (PBS), the milk matrices showed the same threshold judging from the appearance of visual staining in the test line of the test strip (10^3 CFU mL⁻¹).

The magnetic signal (MS) intensities of T line of the experiments mentioned above were measured by MAR (Magnetic Assay Reader), and the results were expressed as relative magnetic units (RMUs). As shown in Fig. 3, the signals grew with the increase of *E. coli* O157:H7 concentrations. A significant linear relationship presented a log-log plot between *E. coli* O157:H7 concentration and MS of T line in the range from 1×10^1 to 1×10^6 , with a linear coefficient of 0.9954. This provides a quantitative method for *E. coli* O157:H7 detection. The calculated detection limit was 32 CFU mL⁻¹ (three times the negative sample signal, 3×65 RMUs), which was lower than the detection limit judging by naked eye (about 10^3 CFU mL⁻¹) and also closer to the infective dose of *E. coli* O157:H7 (50 – 100 cells).

The results of the milk matrix assays were also analyzed by MS intensity. As can be seen from Fig. 3, MS intensities still

Fig. 3. Standard curves of the ICA results determined by magnetic intensity shown in a log-log plot. X and Y axis are all expressed on logarithm. Note: MS, magnetic signal; RMUs, relative magnetic units.

presented linear relationship with *E. coli* O157:H7 concentration in log-log plot. The linear range for quantitative measure has been narrowed down to 10^2 – 10^5 CFU mL⁻¹, although the MS intensities indicated the detection limit of 100 CFU mL⁻¹, the same level with that in PBS. The transport velocity of the reagents across the membrane also somewhat slowed down in the assays for milk samples. The results showed that presence of the milk matrix exhibited interference with ICA. Nevertheless, the development of the color in the test zone and control zone can be completed in 20 min. Taken together, this ICA strip can be applied to screen *E. coli* O157:H7 in water or polluted milk. The results can be determined by both visual detection and MS intensity. The latter one can quantitatively measure the *E. coli* O157:H7 concentrations at the same time with a lower detection limit.

The capacity of the sandwich reaction was determined by both capture and detection antibodies with high antigen specificity and the sensitive labeling methods. The detections of the bacteria are often poor due to single targeting of the bacteria antigen using mAb both as capture and detection antibodies. For instance, a sandwich ELISA using a mAb to the LPS of *E. coli* O157 has a detection limit of 10^5 CFU mL⁻¹ (Kerr *et al.* 2001). Sunwoo *et al.* use IgY as a detection antibody showed the detection limit of 10^3 CFU mL⁻¹ by ELISA (Sunwoo *et al.* 2006). The ICA developed using IgY as the detection antibodies here can achieve a detection limit of 10^3 CFU mL⁻¹ without pretreatment of the milk samples, which can be also analyzed by MS intensity with a detection limit of 10^2 CFU mL⁻¹. This procedure is more rapid and did not use any devices or dangerous compounds and provides an alternative semiquantitative measurement with lower limit of detection at the same time. Therefore, this method is suitable for on-spot quality test of raw milk purchases.

Specificity of the ICA system The cross-reactivity of the ICA strips with four kinds of *E. coli* O157:H7 strains and other related bacteria were tested. Table 2 showed the observed results. Six *E.*

Table 2. Specificity of the ICA strips.

Strain	Number of strains	Results of ICA (Cross-reactivity)
<i>E. coli</i> O157:H7 (ATCC 43895)	1	++
<i>E. coli</i> O157:H7 ^a	6	++
Other STEC <i>E. coli</i> ^b	4	+
Non-pathogenic laboratory <i>E. coli</i> ^c	7	-
<i>Salmonella</i> ^d	2	-
<i>Staphylococcus aureus</i> ^e	2	-

++: positive result; +: positive result; -: negative result

^a*E. coli* O157:H7 strains : ATCC 43888, ATCC 43889, CMCC44828, NCTC 12900, 99X131 and 99X132 JS-CDC

^bOther STEC *E. coli* : O26:H11, O111, O103:H2, O145

^cLab strains : BL21 DE3, BL21RIPL, JM109, CMCC44102, ATCC25922, CMCC44103, CMCC44350

^d*Salmonella* : *S. typhimurium* ATCC 13311, *S. urbana*.

^e*Staphylococcus aureus* : CMCC26001, CMCC26003

Table 3. Results of ICA for spiked samples.

Concentration of <i>E. coli</i> O157:H7 (CFU mL ⁻¹)	Visual result	FN rate (%) ^a	Mean MS of T line (RMUs)	SD of MS ^b
10 ²	-	95	285.35	±21.65
10 ³	+	10	860.05	±34.25
10 ⁴	+	0	2550.85	±54.60
10 ⁵	+	0	8669.05	±80.50

+: positive; -: negative.

^aFN rate, negative rate was calculated as: %FN=(fn/tp)×100, where fn are negative test samples; tp are truly positive samples.

^bSD standard deviation.

coli O157:H7 strains could be detectable by the ICA. Four other STEC strains showed weak cross-reactivity. However, among seven *Non-pathogenic laboratory E. coli* strains tested, none of them showed cross-reactivity. The interferences of the other bacteria examined to the assays were negligible, especially for the *Staphylococcus aureus* (*S. aureus*) which contains protein A in the cell wall capable of covalently binding to the Fc region of IgG of most mammalian species with high affinity (Xiong *et al.* 2014) but not IgY. In addition, it have been reported that some specific IgY antibodies can be raised in egg yolk (Ferreira Junior *et al.* 2012). As a result, using as a detecting antibody, IgY can avoid the false positive results in application.

Repetition and Stability of ICA Spiking samples were analyzed by ICA and the results were observed both by naked eyes and MAR (Table 3). The consistent results were obtained in the same levels with twenty replications, showing good reproducibility and strip-to-strip performance either analyzed by color or by MAR.

The stability of MS was evaluated by measuring the ICA strip by MAR on the first and 12th weeks since sample addition. The MS intensity presented good stability with a slightly decline after 12-week exposure at room temperature. The results were in coincide with what has been reported by Xu *et al.* (Xu *et al.* 2009). The results supported the idea that MS of this ICA test can remain relatively stable in a long term, compared with signal based on

optical measurement.

Conclusions

In this study, it has been demonstrated that the super-paramagnetic nanoparticles with red color (GM) can be employed for the development of ICA strip. An IgY specific to *E. coli* O157:H7 was prepared and labeled by GM to develop a sandwich ICA for *E. coli* O157:H7. The ICA was optimized and testified with a limit of 10³ CFU mL⁻¹ for visual detection. Using MS intensity detection, the ICA can quantitatively measure *E. coli* O157:H7 concentration with a detection limit of 32 CFU mL⁻¹ in PBS and 10² CFU mL⁻¹ in pretreatment-free milk samples. The assay could be completed in less than 20 min. The visual detection is fit for supervising the water environment or rapid screening of milk on spot of purchases and the result are also can be preserved and quantitatively reassay by MS intensity.

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