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Multiplex lateral flow immunoassay for five antibiotics detection based on gold nanoparticle aggregations

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A new immunochromatographic assay (ICA) was developed for the simultaneous screening of five antibiotics that can coexistence in milk, namely lincomycin (LIN), gentamicin (GEN), kanamycin (KAN), streptomycin (STR), and neomycin (NEO), using five corresponding monoclonal antibodies (mAbs). The five mAbs were conjugated to colloidal gold nanoparticles (AuNPs), forming AuNP-labeled antibodies, which were placed in a microtiter well after freeze-drying; the five antigens were immobilized separately on five test lines to align with their corresponding AuNP-labeled antibodies. Using this method, the cutoff values for the strip test in milk were 25 ng/mL for LIN, 25 ng/mL for GEN, 50 ng/mL for KAN, 50 ng/mL for STR, and 100 ng/mL for NEO, which are below the maximum residue levels set by the European Union. Based on the strip reader, the multiplex strip could detect the concentrations of LIN, GEN, KAN, STR, and NEO as low as 2.5 ng/mL, 2.5 ng/mL, 2.5 ng/mL, 2.5 ng/mL, and 5 ng/mL in milk, respectively. The accuracy and reproducibility of the assay were acceptable when tested on food samples. In conclusion, our ICA strip is useful for the rapid and high-throughout screening of antibiotics in food and environments on-site.

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1. Introduction

Antibiotics are widely used in veterinary treatment. However, their use can have numerous negative effects, such as enhanced drug resistance and contamination of the environment, and are a threat to public health (Van Boeckel et al. 2015; Wang et al. 2015; Zhang et al. 2015).[1-3] Lincomycin (LIN) belongs to the lincosamide class of antibiotics, and was derived from the bacterium, *Streptomyces lincolnensis.* This antibiotic is often injected into dairy cattle via the intramuscular or intramammary routes to protect against Gram-positive bacteria (Burkin and Galvidis 2010).[4] Genomic damage can be induced after long-term or high-level exposure of LIN, and can be enhanced by concomitant use of other antibiotics (Hagenbuch and Pinckney 2012; Rocco et al. 2012).[5,6] Gentamicin (GEN), kanamycin (KAN), streptomycin (STR) and neomycin (NEO) are often found as antibiotic residues in milk, and are grouped in a subclass of aminoglycosides (AMGs) (Tao et al. 2012).[7] GEN is used to treat both Gram-positive and -negative bacteria by parenteral or oral injection in cattle (Chen et al. 2008a).[8] KAN, produced by *Streptomyces kanamyceticus*, can inhibit the growth of both Gram-positive and -negative bacteria (Park et al. 2011; Zaunbrecher et al. 2009),[9,10] while STR is used for treating Gram-negative bacteria, and works by blocking the synthesis of protein (Mølbak et al. 1999).[11] Neomycin (NEO), a broad-spectrum antibiotic, is injected orally to treat gastrointestinal infections and via the intramammary route to treat mastitis in cattle (Gaudin et al. 2005).[12] Excessive and long-term usage of AMGs may lead to ototoxicity and nephrotoxicity (Gaudin et al. 2005).[12] The presence of residues in milk is easily detected if the suggested withdrawal time is not respected, and is particularly obvious after intramammary or intrauterine injections rather than intravenous or intramuscular injections (McGlinchey et al. 2008; Smyth et al. 2005).[13,14] Therefore, the following maximum

residue levels (MRLs) in milk were established by the European Union (EU): 150 μg/kg for LIN, 100 μg/kg for GEN, 150 μg/kg for KAN, 200 μg/kg for STR, and 1500 μg/kg for NEO (Regulation 2009).[15]

Many analytical methods have been developed for monitoring the contamination levels of these five antibiotics in milk, including microbiological assays, instrument methods, and immunoassay methods (Stead 2000).[16] Microbiological assays generally lack sensitivity and specificity (Adams et al. 1998),[17] while instrument methods, including high-performance liquid chromatography, liquid chromatography–mass spectrometry, and liquid chromatography–tandem mass spectrometry (Han et al. 2015; Tao, et al. 2012; Turnipseed et al. 2008),[7,18,19] are costly, time consuming, require complicated sample preparation, and are unsuitable for field applications, despite the advantages of higher sensitivity and multiplex detectability (Grebe and Singh 2011).[20] Previous studies have reported the use of immunoassay methods such as ELISA and biosensor methods for monitoring antibiotic residues (Burkin and Galvidis 2010; Chen, et al. 2008a; Chen et al. 2008b; Chen et al. 2013; Schoukroun-Barnes et al. 2014; Taranova et al. 2015; Zhou et al. 2014; Liu, et al. 2011).[4,8,21-26] Among these methods, antibody-based multicomponent strip sensors are a particularly good method for field use due to their time efficiency and simple preparation compared with the complex steps required for ELISA such as washing and incubation (Guo et al. 2010; Xing et al. 2015; Ren et al. 2014).[27-29] For this purpose, an immunochromatographic assay (ICA) has been proposed for detecting these antibiotic residues in milk. Several studies have been published on the detection of these five antibiotic residues using ICA methods (Byzova et al. 2011; Cao et al. 2015; Chen et al. 2008b; Jin et al. 2005; Jin et al. 2006).[21,30-33] However, to our knowledge, there have been no reports of ICA for the simultaneous detection of these five analytes using different monoclonal

antibodies (mAbs). In this paper, we aimed to develop a one-step ICA for the rapid and simultaneous screening of LIN, GEN, KAN, STR, and NEO, and to assess its application in spiked milk.

2. Materials and methods

2.1. Reagents and apparatus

LIN, GEN, KAN, STR, and NEO were purchased from J&K Scientific (Shanghai, China). Goat anti-mouse immunoglobulin (IgG) antibody was supplied by Jackson ImmunoResearch Laboratories (West Grove, PA, USA). All buffer solutions were prepared with ultrapure water from a Milli-Q Ultrapure System (Millipore Corporation, Bedford, MA, USA). All other reagents and chemicals were obtained from the National Pharmaceutical Group Chemical Reagent Co., Ltd. (Shanghai, China). The anti-LIN mAb, anti-GEN mAb, anti-KAN mAb, anti-STR mAb, and anti-NEO mAb were produced in our laboratory (Xu et al. 2011).[34]

The polyvinylchloride (PVC) backing material, the sample pad (glass fiber membrane, GL-b01), the absorbance pad (H5079), and the conjugate pad (Ahlstrom 8964) were obtained from JieYi Biotechnology Co., Ltd. (Shanghai, China). The nitrocellulose (NC) membrane was purchased from Millipore Corporation. The CM4000 Guillotine Cutting Module and the Dispensing Platform were obtained from Shanghai Kinbio Tech Co., Ltd. (China). The BioDot TSR3000 Membrane Strip Reader was supplied by Gene Company Limited (Shanghai, China).

2.2. Preparation of the hapten–protein conjugates

The hapten–bovine serum albumin (BSA) conjugates (NEO–BSA, KAN–BSA, STR–BSA, and GEN–BSA) were synthesized using the glutaraldehyde method, as

described previously (Xu et al. 2014).[35] Briefly, 5.37 μL glutaraldehyde solution (25%) diluted 10 times with 0.01 M phosphate buffered saline (PBS, PH7.4) was added dropwise into 13.4 μmol AMGs (GEN, KAN, STR, and NEO) dissolved in 600 μL 0.01 M PBS. After stirring for 10 min at room temperature, the solution mixture was slowly added to 10 mg BSA dissolved in 3.4 mL 0.01 M PBS. After reacting for 40 min at room temperature, 16 mg sodium borohydride was added. The mixture was allowed to react for 2 h at 4 °C, and then dialyzed against 0.01 M PBS for 3 days.

The LIN conjugate (LIN–BSA) was prepared using the following method. LIN succinate (LIN-HS) was conjugated to BSA by the 1-ethyl-3-(3-dimethylaminopropyl)- carbodiimide (EDC)/N-hydroxysuccinimide (NHS) method (Burkin and Galvidis 2010).[4] Briefly, 3.3 mg EDC (17.2 μmol) and 1.5 mg NHS (13 μmol) were added to LIN-HS (8.9 μmol) dissolved in 400 μl N, N-dimethylformamide (DMF). The mixture was reacted for 4 h at room temperature and then slowly added to 10 mg BSA dissolved in 5 mL 0.01 M borate buffer (BB, PH 8.4). The mixture was maintained at room temperature overnight, followed by dialysis against 0.01M PBS for 3 days.

2.3. Preparation of the immunochromatographic strip and its characterization

The AuNP solution was prepared as previously reported (Xu et al. 2012),[36] with some modifications. Briefly, 10 mL of 1% trisodium citrate solution was rapidly added to 500 mL of 0.01% $HAuCl_4$ solution, and then boiled with constant stirring. The reaction solution changed to the color of red wine within around 1 min, and then we continued boiling the mixture for another 5 min. The solution was then cooled to room temperature and stored at 4 °C for following experiment.

The anti-AMGs/LIN mAbs produced in our laboratory were purified by the caprylic acid–ammonium method, and then evaluated by indirect competitive ELISA and our single strip method. The indirect competitive ELISA was performed as previously described (Peng et al. 2015).[37] Briefly, 100 μL of AMGs/LIN–BSA diluted with 0.05 M carbonate buffer (CB) were coated onto 96-microwell plates for 2 h at 37 °C. Then, the plates were washed three times with 0.01M PBS with 0.05% Tween 20 (PBST) and blocked with 0.05 M CB with 0.2% w/v gelatin for 2 h at 37 °C. After being washed thrice again, 50 μL of different concentrations of the analytes in PBS and 50 μL mAb dissolved in antibody diluent (0.01 M PBS containing 0.05% v/v Tween 20 and 0.1% w/v gelatin) were added to each well. After incubation for 30 min at 37 °C, the plates were washed with PBST, and 100 μL of peroxidase-labeled goat anti-mouse IgG (diluted 3000 times with the antibody diluent) were added to each well and incubated for 30 min at 37 °C. After being washed with PBST, 100 μL of 3,3',5,5'–tetramethylbenzidine substrate was added, and the reaction was stopped by the addition of 50 μL of 2 M H_2SO_4, followed by incubation at 37 °C for 15 min. The optical density was then read by the microplate reader at 450 nm.

The AuNP-labeled antibodies were prepared using the following method (Liu et al. 2014).[38] First, the pH of the AuNP solution (1 nM) was adjusted to 8 with K_2CO_3. Then, the purified mAb, which was dissolved in ultrapure water, was added dropwise to 10 mL of the above-mentioned AuNP solution. After gentle stirring for 30 min, 0.5 mL of 10% (w/v) BSA was added to block the unreacted sites. After 1 h, the mixture was centrifuged twice at 8000 *g* for 20 min. Finally, the precipitate was resuspended in 1 mL of boric acid buffer solution (0.002 M boric acid, 0.2% BSA, and 0.02% NaN_3).

The immunochromatographic strip was assembled as shown in Figure 1A. The NC membrane was glued to the middle of the PVC support plate, and the sample pad and absorbent pad were glued to the bottom and upper regions, respectively. The goat anti-mouse IgG antibody and antigens were sprayed onto the NC membrane part of the strip as the control and test lines (C line and T lines, respectively) at a speed of 1 μL/cm using the rapid test dispenser platform (HM3035). The distance between the T lines and C line was 5 mm. After drying at 37 °C for 2 h, the strips were cut to the specification of 3 mm × 60 mm using a guillotine cutter, and then stored in a sealed bag at room temperature together with the mAb–AuNP conjugates, which were freeze-dried using a vacuum freeze dryer. The antigens specific to the five different kinds of antibiotics (NEO, STR, KAN, GEN, and LIN) were evenly distributed on the NC membrane in the tomographic flow direction to form five separate T lines for simultaneous detection. In this study, a 200 μL sample solution containing all five analytes (LIN, GEN, KAN, STR, and NEO) at different concentrations was added to the mAb–AuNP conjugates in the microtiter wells. The optimal concentrations of the antigens synthesized with the standard bioconjugation techniques were 0.5 mg/mL for LIN–BSA, GEN–BSA, KAN–BSA, and STR–BSA, and 1 mg/mL for NEO–BSA on the test lines. After reacting for 5 min at room temperature, strips were then added to the mixtures. Under these conditions, the antigen-antibody complex was formed. The amount of complex was proportional to the amount of analyte in the original sample. These complex, in solution, were contacted with the strip sample pad and migrates into the NC membrane. The five coating reagents and the goat anti-mouse IgG was sprayed on the NC membrane and formed the five test lines and control line before. If there is no analyte in the sample solution, the GNP-labeled antibodies bind to the immobile coating antigens on the T lines (TL-1, -2, -3, -4, and -5) and the goat anti-mouse IgG on the C line separately. This result was considered negative. If a certain amount of analytes exist in the sample solution, free analytes (in the sample solution) would combine with the corresponding mAb-AuNP conjugates. Less mAb-AuNP conjugates would combine with the immobile coating antigens and the intensity of the T lines will decrease. If all the five analytes concentration is high

enough, the five T lines will all disappear. Excess reagents that cross the NC membrane become entrapped in an absorbent pad.

(Figure 1)

2.4. Sensitivity of the multiple strips

Five kinds of mixed standards were prepared for detecting the visual detection limit of the multiple strips. LIN, STR, KAN, and GEN were diluted to 0, 2.5, 5, 10, 25, and 50 ng/mL, and NEO was diluted to 0, 5, 10, 25, 50, and 100 ng/mL. PBS (100 μL) with and without the five analytes was added to the microtiter wells with the mAb–AuNP conjugate mixtures. After 5 min reaction time between the analytes and the mAb–AuNP conjugates, a strip was put into each microporous well. The solution moved along the NC membrane with the help of capillary action and aggregated on the test and control lines due to the antigen–antibody reaction. After 10 min, the results were evaluated with the naked eye. The anti-AMGs/LIN mAb–AuNP conjugates freely migrated into the NC membrane and combined with the immobilized AMGs/LIN–BSA antigen on the test zones in the absence of the five analytes in the sample solution. Therefore, five visible red lines were formed, as showed in Figure 1A. In contrast, if the concentrations of the five analytes reached certain values, the mAb–AuNP conjugates would first bind to the corresponding free analytes in the sample; thus, few or no mAb–AuNP conjugates would migrate and combine with the immobilized AMGs/LIN–BSA antigen. Thereafter, a weakly colored line or no red line was then observed in the corresponding zone as showed in Figure 1B. All the uncombined mAb–AuNPs conjugates gathered on the control line, forming a deep red

band after a complete reaction between the mAbs–AuNP conjugates and the goat-mouse IgG antibody.

2.5. Assay of milk samples with the multiplex strip

Milk samples were used to estimate the practicability of the multiplex strip. Different concentrations of LIN, GEN, KAN, STR, and NEO were spiked in negative milk samples previously confirmed by ELISA. The analyses were repeated 20 times for each concentration (n = 20).

3. Results and discussion

3.1. Preparation of antibody–AuNP conjugates

AuNPs (15±1 nm) contributes to the plasmon peak absorption at 520 nm (Figure 2). The antibody–AuNP conjugates formed due to the high electron density of the gold particles and the fact that their surfaces can bind to biological macromolecules such as antibodies (Zhao et al. 2014; Nara et al.2010).[39,40] The pH of the AuNP solution and the amount of the mAbs were optimized during the preparation of the antibody–AuNP conjugates. Briefly, different volume of 0.1 M K_2CO_3 (2μL, 4μL , 8μL , 10μL) was added to tubes containing 1 mL AuNP solutions to determine the optimal pH value. 20 μL of 1mg/mL mAb solution was then added and incubated for 10 min at room temperature. Then, 100 μL of 10% NaCl solution was added dropwise to each tube. Based on the results of our experiment, the color of the mAb-AuNP conjugates were showed no color change through the UV spectrum and the naked eye observation when 4 μL of 0.1 M K_2CO_3 was added to 1 mL AuNP solution to adjust the pH (pH=7.2). 2 μL K_2CO_3 will result in low pH (pH=6.8) and unstable mAb-AuNP conjugates. The color of conjugates were commonly bluish violet and the AuNP will aggregate after 10% NaCl were added or after centrifugation. 8 μL K_2CO_3 (pH=7.8) or 10 μL K_2CO_3 (pH=8.2) could be used but not recommended because the antibody

labelling efficiency will decrease as the charge of antibody will be more negative at pH above its isoelectric point (Table S1). Therefore, the addition of 4 μL of 0.1 M K_2CO_3 to each 1 mL AuNP solution optimized the pH value. To determine the optimal amount of mAb to conjugate to the gold nanoparticles, a gradient concentration of each mAb solution was added into 1 mL AuNP solution adjusted with 4 μL of 0.1 M K_2CO_3. After 10 min, 100 μL of 10% NaCl solution was added dropwise and incubated for 10 min. The optimal amount of mAb to conjugate to the gold nanoparticles was in excess of 10% of the lowest amount of mAb, which was effective for preventing aggregation of the colloidal solution. The results showed that the optimal concentrations for the five mAb–AuNP conjugates were 8 μg /mL for anti-LIN and anti-KAN, 10 μg/mL for anti-GEN and anti-STR, and 15 μg/mL for anti-NEO mAbs. To reduce nonspecific reactions, 50 μL of 10% BSA was added to 1 mL of each mAb–AuNP conjugate with 1 h of slight shaking to block any unconjugated sites.

(Figure 2)

(Table S1)

3.2. Optimization of the immunochromatographic strip

High sensitivity and specificity of the antibodies and the corresponding antigens play key roles in the performance of this multiplex strip. In our study, five highly sensitive antibodies were prepared and the half maximal inhibitory concentration values were confirmed with indirect ELISAs and the single strips (Table S2); Figure 3 shows the single test immunochromatographic strips for LIN, GEN, KAN, STR, and NEO in milk. On the control lines, the optimal concentration of goat anti-mouse IgG antibody

was determined to be 0.5 mg/mL. Under these optimized conditions, the developed ICA had clear bands, with high sensitivity.

(Table S2)

(Figure 3)

3.3. Evaluation of the multiplex strip

Five analytes at different concentrations were mixed in PBS to generate standards for evaluating the multiplex strip. For the assay, the end of the test-strip sample pad was placed into the microtiter wells and 100 μL of extracts with and without standards was added to the wells with the five freeze-dried antibody–AuNP conjugates. The 100 μL extracts in different wells contained different levels of LIN, GEN, KAN, STR, or NEO as shown in Figure 4. The standards were first bonded with the antibody–AuNP conjugates in the solution in the wells, and then, driven by capillary forces, migrated from the sample pad to the absorbent pad by way of the hapten–protein conjugates on the test lines and goat anti-mouse IgG antibody on the control line. The whole process took 5 min. As shown in Figure 4A, the intensity of the color of the test lines decreases as the concentration of the analytes increases. Based on visual inspection, the cutoff levels for this method in PBS were 50 ng/mL for LIN, 10 ng/mL for GEN, 10 ng/mL for KAN, 20 ng/mL for STR, and 20 ng/mL for NEO. The optical density profiles of the test lines (TL-1, -2, -3, -4, and -5) and the control line (CL) were recorded by strip reader and showed in Fig. 4C. The weakly positive results (the test line is clearly lighter than the negative result but has not disappeared) indicate that the multiplex strip works when the concentrations of LIN, GEN, KAN, STR, and NEO are 5 ng/mL, 1 ng/mL, 1 ng/mL, 2.5 ng/mL, and 2.5 ng/mL, respectively.

(Figure 4)

Similarly, milk samples spiked with different concentrations of the five standards were tested using our multiplex strip. As shown in Figure 4B, the cutoff levels for this method in milk were 25 ng/mL for LIN, 25 ng/mL for GEN, 50 ng/mL for KAN, 50 ng/mL for STR, and 100 ng/mL for NEO, which are lower than the MRLs in milk set by the EU. The optical density profiles of the test lines (TL-1, -2, -3, -4, and -5) and the control line (CL) were recorded by strip reader and showed in Fig. 4D. The weakly positive results indicate that the multiplex strip works when the concentrations of LIN, GEN, KAN, STR, and NEO are 2.5 ng/mL, 2.5 ng/mL, 2.5 ng/mL, 2.5 ng/mL, and 5 ng/mL, respectively. In the milk matrix, antibody could interact with LIN more sensitively and the detection limit was decreased 2-fold. The detection limits for GEN, KAN, STR, and NEO was unchanged or increased.

Table 1 shows no cross-reactivity of these five analytes by ELISA. This can be attributed to the different structures of the analytes shown in Table S2 and the specific antibodies we prepared.

(Table 1)

The accuracy and reproducibility of the developed ICA strips were further evaluated by spiking four different concentrations of the analytes into negative milk samples. The semi-quantitative results in Table S3 indicate that the ICA results are consistent with the spiked analyte concentrations based on visual inspection. The

quantitative results in Table S4 indicate the ICA good stability and application with the recoveries ranging from 86.2% to 121.7%. Concordant results were obtained for 20 replicates of the strip test.

(Table S3)

(Table S4)

Thus, our ICA strip method could be a useful tool for the multiple screening of LIN, GEN, KAN, STR, and NEO residues in milk.

4. Conclusions

In the present study, a newly developed ICA was used to screen five antibiotics that can coexist in milk using five corresponding mAbs. Highly sensitive and specific antibody–antigen systems ensure the sensitivity and specificity of the developed ICA. The lack of complicated sample pretreatment, naked eye analysis within 10 min, and ease of use, makes the strip suitable for on-site analysis.

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Captions:

Fig. 1 Schematic illustration of the developed ICA.

Fig. 2 Characterization of the AuNPs solution (A) TEM images (B) UV-vis spectra.

Fig. 3 Single test immunochromatographic strips for LIN, GEN, KAN, STR and NEO in milk

Fig. 4 Typical photo image of detection five analytes by the developed ICA (A) in PBS; (B) in milk

Table 1. Cross-reactivity of five analytes by ELISA

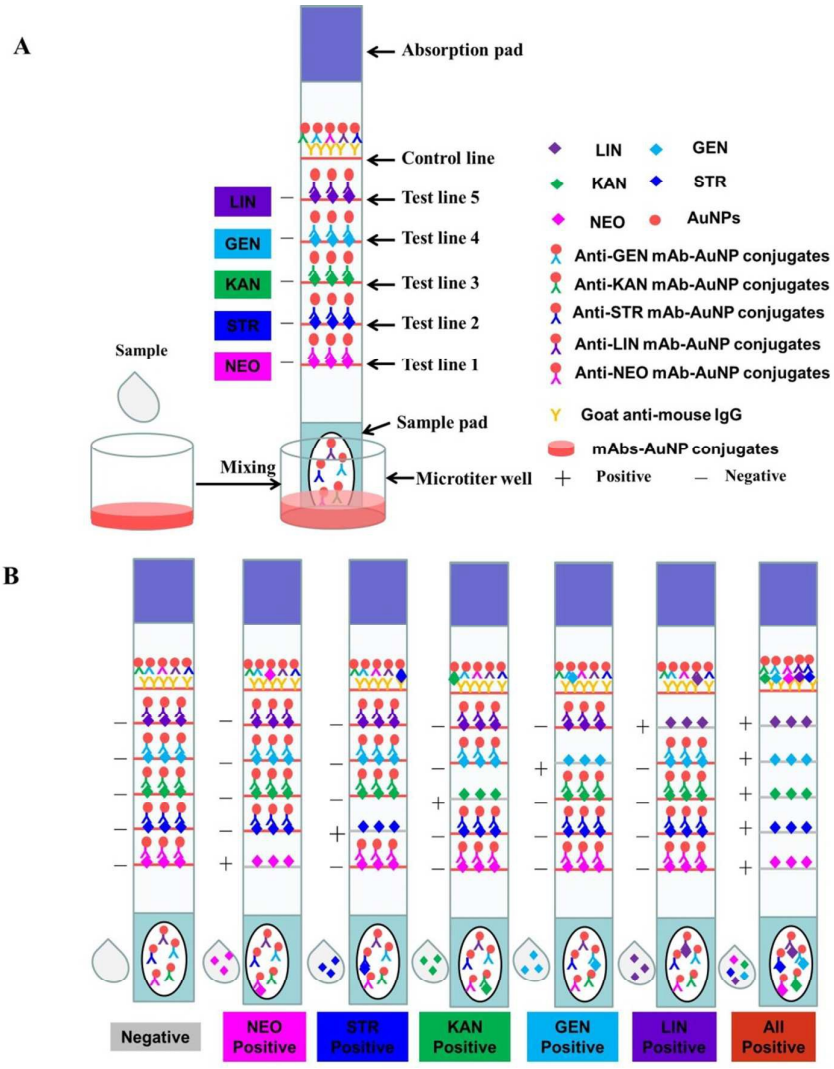

Fig. 1 Schematic illustration of the developed ICA.

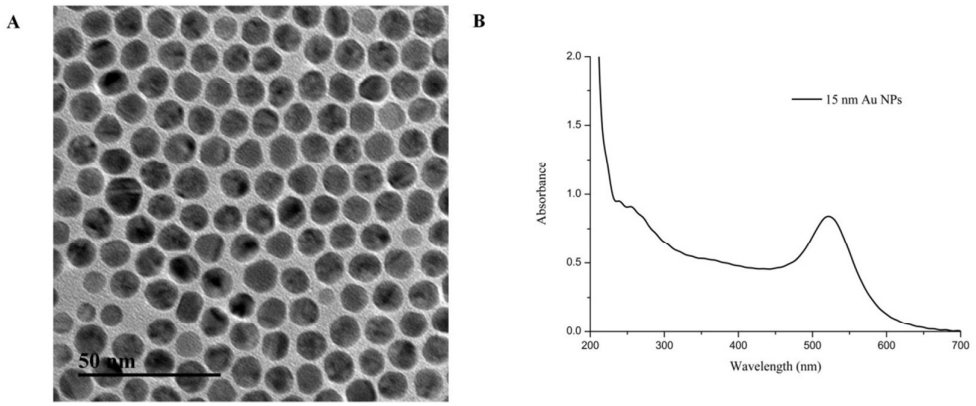

Fig. 2 Characterization of the AuNPs solution (A) TEM images (B) UV-vis spectra.

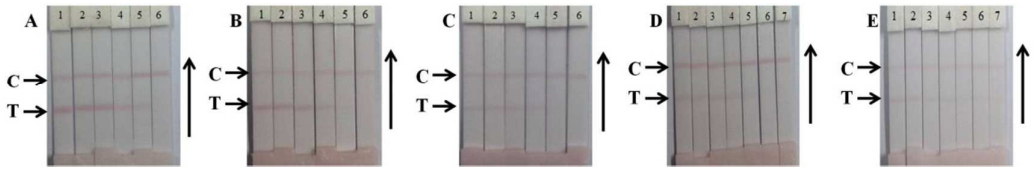

Fig. 3 Single test immunochromatographic strips for LIN, GEN, KAN, STR and NEO in milk (A) LIN: 1 = 0 ng/mL, 2 = 0.1 ng/mL, 3 = 0.25 ng/mL, 4 = 0.5 ng/mL, 5 = 1 ng/mL, and 6 = 2.5 ng/mL (B) GEN:1 = 0 ng/mL, 2 = 0.25 ng/mL, 3 = 0.5 ng/mL, 4 = 1 ng/mL, 5 = 2.5 ng/mL, and 6 = 5 ng/mL (C) KAN: 1 = 0 ng/mL, 2 = 0.25 ng/mL, 3 = 0.5 ng/mL, 4 = 1 ng/mL, 5 = 2.5 ng/mL, and 6 = 5 ng/mL (D) STR:1 = 0 ng/mL, 2 = 0.5 ng/mL, 3 = 1 ng/mL, 4 = 2.5 ng/mL, 5 = 5 ng/mL, 6 = 10 ng/mL, and 7 = 12 ng/mL (E) NEO: 1 = 0 ng/mL, 2 = 0.5 ng/mL, 3 = 1 ng/mL, 4 = 2.5 ng/mL, 5 = 5 ng/mL, 6 = 10 ng/mL, and 7 = 15 ng/mL

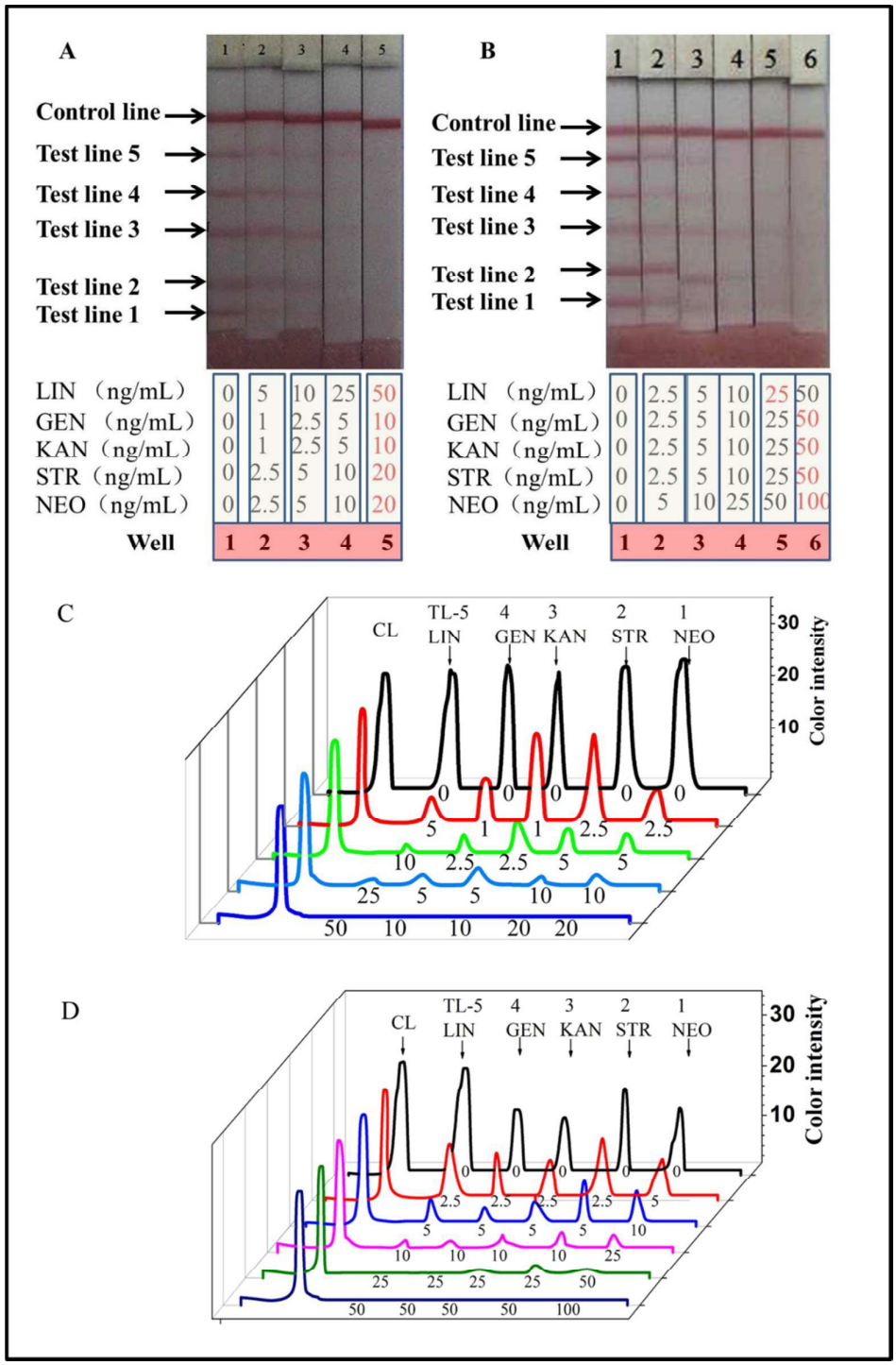

Fig. 4 Typical photo image of detection five analytes by the developed ICA in PBS (A) and milk (B) and optical density profiles of the Test lines (TL-1,2,3,4,5) and Control lines (CL) of detection results in PBS (C) and milk (D).

Table 1. Cross-reactivity of five analytes by ELISA

Analytes	Analogues	Cross-reactivity (%)
LIN	GEN	<0.01
	KAN	<0.01
	STR	<0.01
	NEO	<0.01
GEN	LIN	<0.01
	KAN	<0.01
	STR	<0.01
	NEO	<0.01
KAN	LIN	<0.01
	GEN	<0.01
	STR	<0.01
	NEO	<0.01
NEO	LIN	<0.01
	GEN	<0.01
	KAN	<0.01
	STR	<0.01