Multiplex Immunogold Chromatographic Assay for Simultaneous Determination of Macrolide Antibiotics in Raw Milk

Xiangmei Li · Kai Wen · Yiqiang Chen · Xiaoping Wu · Xingyao Pei · Qi Wang · Ailing Liu · Jianzhong Shen

Abstract A rapid, simple, reliable, and sensitive immunogold chromatographic assay (IGCA) was described for simultaneous determination of four macrolide antibiotics (erythromycin, spiramycin, tilmicosin, and tylosin) in raw milk. Three antigens were immobilized as three test lines on the nitrocellulose membrane, which enable the simultaneous determination on a single test strip. Samples were detected directly without treatment; the entire testing process was completed within 10 min. The visual detection limits for erythromycin, spiramycin, tilmicosin, and tylosin were 5, 5, 10, and 20 ng/mL, respectively. Sixty blind raw milk samples were analyzed by both IGCA and liquid chromatography–tandem mass spectrometry; the results showed a good correlation between the two methods. The results demonstrate that the developed method could provide a rapid and effective approach for the onsite determination of multi-macrolide antibiotics residues in a large number of samples.

Keywords IGCA · Macrolide antibiotics · Test strip · Raw milk

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Introduction

Macrolides are a group of antibacterial compounds that display highly active against Gram-positive bacteria and mycoplasma species (Zhang et al. 2013); owing to their high oral bioavailability and their ability to accumulate intracellularly in many tissues and organs (Maher et al. 2008), they are widely used in medical and veterinary practices to prevent and treat diseases in swine, poultry, cattle, and sheep. For example, tylosin (TYL) and tilmicosin (TIM) are special anti-infection and growth-promoting antibiotics for livestock; erythromycin (ERY) is used to treat clinical or subclinical mastitis in lactating cows (Wang et al. 2006); and spiramycin (SPI) is extensively used to treat respiratory infections which are caused by Gram-positive bacteria and some Gram-negative bacteria.

In China, the phenomenon of irrational use of macrolides is widespread because of low prices and good effect, such as incorrect use or non-compliance with the withdrawal period, etc. This easily leads to the presence of macrolide drug residues in the food of animal origin including raw milk. Nevertheless, the residue of drugs could cause toxic effects, for example, allergic reactions in hypersensitive individuals, or may indirectly cause problems through the induction of resistant strains of bacteria (Horie et al. 1998). In order to guarantee safe food and protect the health of consumers, the maximum residue limits (MRLs) for macrolides in animal foods have been established by many countries and regions. The requirement of MRL for macrolides in milk is the most stringent among all animal foods. The USA, the European Union (EU), and China have set the MRLs for TYL, TIM, SPI, and ERY in milk to be 50, 50, 200, and 40 μg/kg, respectively (Ministry of Agriculture of the People's Republic of China 2002). Therefore, it is extremely important and also necessary to monitor macrolides in animal foods, especially in milk.

Various analytical methods have been published to analyze for macrolides, such as microbiological assay (Breier et al. 2002), thin layer chromatography (TLC) (Kibwage et al. 1983), high-performance liquid chromatography (HPLC) (Maher et al. 2008; Horie et al. 1998), liquid chromatography–mass spectrometry (LC–MS) (Billedeau et al. 2003; Berrada et al. 2008), LC–MS/MS (Wang et al. 2006; Lucchetti et al. 2005; Deubel et al. 2006), etc. Microbiological assay and TLC can be used for screening analysis; however, they are time consuming and lack sensitivity. Although the high specificity and sensitivity can be achieved by HPLC, LC–MS, and LC–MS/MS methods, they require skilled technicians, expensive instrumentation, and complex sample pretreatment, which may not always be available for routine monitoring. In comparison, the immunoassay is a simple, low cost, and sensitive method for screening purposes because of its high-throughput and rapid turnaround time. Enzyme-linked immunosorbent assay (ELISA) and immunogold chromatographic assay (IGCA) are the most commonly used immunoassay. IGCA may be an alternative to the ELISA, which still requires microplate reader and labor-intensive operations including incubation, washing, and enzymatic reactions to obtain the experimental results; the IGCA does not require expensive equipment, skilled personnel, tedious sample pretreatment, and the results can be quickly obtained within 10 min. Now, the use of IGCA for multi-residue analysis is a trend (Heinrich et al. 2013; Le et al. 2013). However, review of the literature reveals that no multiresidue IGCA method have been reported until now for simultaneous determination of four macrolides in raw milk, and the detection of the four drugs was completed on a single test strip by three test lines. Therefore, the goal of this study was to develop a simple, rapid, sensitive, and reliable IGCA method to simultaneously detect four macrolides residues in raw milk.

Materials and Methods

Reagents and Materials ERY, SPI, TYL and TIM, bovine serum albumin (BSA), goat anti-mouse IgG, and chloroauric acid (HAuCl$_4$·4H$_2$O) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The anti-ERY monoclonal antibody (mAb), the anti-SPI mAb, the anti-TIM polyclonal antibody (pAb), ERY-BSA conjugate, SPI-ovalbumin (OVA) conjugate, and TIM-BSA conjugate were obtained from WDWK Biotech Co. (Beijing, China). Purified water was obtained using a Milli-Q water purification system (Millipore, Bedford, MA). The nitrocellulose (NC) membranes, including Millipore (HF13520s25), Sartorius (Unisart CN140), and Mdi (90CNPH-N-SS40), were purchased from Millipore Corporation (Bedford, MA), Sartorius Stedim Biotech (GmbH, Germany), and Advanced Microdevices Pvt. Ltd. (Ambala, India), respectively. The sample pad

(CFKJ-0328) and the absorbance pad (CH37K) were supplied by Shanghai Liangxin Co. Ltd, (Shanghai, China). The microtiter plates were supplied by Guangzhou JET BIOFIL Co. (Guangzhou, China). Other chemical substances were purchased from Beijing Chemical Reagent Company (Beijing, China).

Apparatus The NanoDrop® ND-1000 spectrophotometer was obtained from Gene Company Limited (Hong Kong, China). The Millrock Magnum Series Freeze-Dryer was purchased from Millrock Technology, Inc. (Kingston, USA). The HQ-60-II vortex mixer was obtained from Beijing North TZ-Biotechnology Development Co. (Beijing, China), and the Lynx 4000 centrifuge was purchased from Thermo Fisher Scientific (GmbH, Germany). The ZX1000 Dispensing Platform and the CM4000 Guillotine Cutting Module were purchased from BioDot Inc. (Irvine, CA, USA).

Preparation of Colloidal Gold Colloidal gold was prepared as follows with some modifications (Li et al. 2011). One hundred milliliter of double-distilled water was heated to boiling under constant stirring, then 2.0 mL of 2 % chloroauric acid solution (*w/v*) was added, when the solution was boiling again, and then 4.5 mL of 1 % trisodium citrate (*w/v*) was immediately fast added. The reaction was boiled for another 10 min, and the solution was then cooled and reconstituted to the initial volume by adding double-distilled water, and then stored at 4 °C for further use.

Preparation of Gold-Labeled Abs In order to quickly and effectively get the best color intensity and sensitivity of the test strip, the optimal pH and Ab concentration for colloidal gold binding to each Ab were adjusted separately by checkerboard titrations (Chen et al. 2008). With gentle stirring, the pH of the colloidal gold solution (20 mL) was adjusted with 540 μL of 0.1 M K$_2$CO$_3$ (*w/v*), then 0.2 mg of anti-ERY MAb, which was diluted in 2.0 mL double-distilled water, was added rapidly to pH-adjusted colloidal gold solution. The mixture was incubated for 10 min at room temperature, and then 400 μL of 20 % BSA (*w/v*) was added dropwise for another 10 min incubation, the solution was centrifuged at 15,428 × *g* for 15 min, and the red gold-labeled MAb precipitate was resuspended with 24 mL of 0.02 M phosphate buffer (PB, pH 7.4) containing 5 % sucrose, 0.5 % BSA, 0.5 % Triton-100, and 0.03 % sodium azide. The preparation of another two gold-labeled Ab were the same to the above description, just the optimal pH and Ab concentration were different. The three prepared gold-labeled Ab solution were stored at 4 °C for further use.

Eighteen microliters of prepared gold-labeled anti-ERY MAb solution, 18 μL of prepared gold-labeled anti-SPI MAb solution, and 12 μL of prepared gold-labeled anti-TYL PAb solution were mixed and added to the microporous, then

the microtiter plates were placed in the Freeze-Dryer to freeze-dry overnight. The lyophilized detection reagents were sealed in foil pouch for further use.

Immobilization of Capture Reagents The three antigens and goat anti-mouse IgG were diluted to 0.5 mg/mL with 0.02 M PBS (2 % sucrose, pH 7.4), respectively. The sprayed volume of each of the above solutions was 0.8 μL/cm, then they were sprayed onto the NC membrane by the ZX1000 Dispensing Platform to form three test lines (test 1 for TIM, test 2 for ERY, and test 3 for SPI) and a control line, respectively. The distance between the two lines was 3.5 mm (Fig. 1). The NC membrane was then dried for 2 h at 37 °C and stored under dry conditions at room temperature until use.

Assembly of the Strip Test Components The components of the test strip including NC membrane coated with capture reagents, absorbent pad, sample pad, back plate, and lyophilized detection reagents (Fig. 1). The sample pad was soaked with 0.02 M PB (0.5 % Tween-20, pH 7.4). The assembly procedure was similar to that described by Chen et al. (2008). The only difference was that there was no conjugate pad. Then the whole assembled plate was cut into 4.5 mm width strips and stored under dry conditions at room temperature.

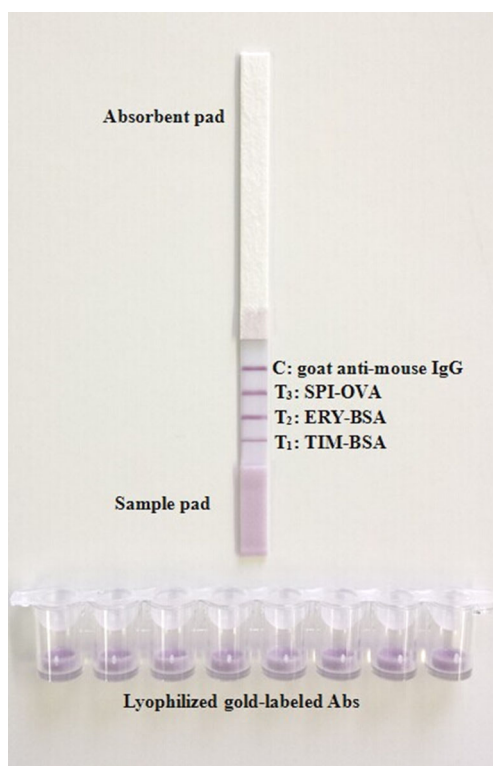*Sample Pretreatment* The raw milk was detected directly without treatment.

Fig. 1 Schematic description of the test strip

Test Procedure The raw milk sample was shaken well before detection, then 200 μL of sample was added into the microporous containing lyophilized detection reagent; the sample was pipetted back and forth to fully dissolve the lyophilized detection reagent. After incubating for 3 min at room temperature, the test strip was inserted into the microporous. With the effect of chromatography role of sample pad, the sample solution containing gold-labeled Abs rapidly flowed to the other end of the strip. The result could be read by visual inspection after 3–5 min. If the concentrations of the four macrolides in sample were below certain values, a part of the detection reagent was trapped by the capture reagent at the test lines and another part of them reacted with the anti-mouse IgG at the control line. Then, the conjugates gathered at the test and control lines to form visible pink bands. Nevertheless, if the concentrations of the four macrolides in the sample were above certain values, each kind of detection reagent was occupied by its corresponding drug, which would then prevent the detection reagent from binding the capture reagent. Then the conjugates of detection reagent and drug passed through the test lines, reacted with the anti-mouse IgG and gathered at the control line. Therefore, just a pink band formed at the control line, the test line signal would decrease to a non-visible line and the results would be positive (Fig. 2).

Evaluation of the IGCA The sensitivity, specificity, stability, repeatability, and reproducibility of the IGCA were evaluated in this work. For the sensitivity of the test strip, series spiked sample concentration of mixed macrolides standard were analyzed by the strip to determine the visible detection limit, which the detection test lines completely disappeared at those concentration. To evaluate the specificity of the strip, ERY, SPI, TIM, and TYL standard solutions were tested for cross-reactivity by the strip test. The stability of the IGCA was evaluated by accelerated stability test, which the strips were stored under 45 °C dry condition for 1 month, then the color intensity and sensitivity of the strips were analyzed on 1, 7, 15, 24, and 30 days, respectively. The repeatability of the IGCA was measured by five duplicate detection for 20 raw milk samples including positive and negative. The reproducibility of the IGCA was evaluated by testing the same samples at different time points.

LC–MS/MS Analysis In order to validate the strip test, four macrolides-spiked raw milk samples were analyzed by a LC–MS/MS method. The procedure of LC–MS/MS was as described in the previous article without modification (Wang et al. 2006).

Blind Samples of Raw Milk The 60 blind samples of raw milk were supplied by the National Veterinary Drug Safety Evaluation Center, Beijing, P. R. China, and all samples were analyzed by both the IGCA and the LC–MS/MS.
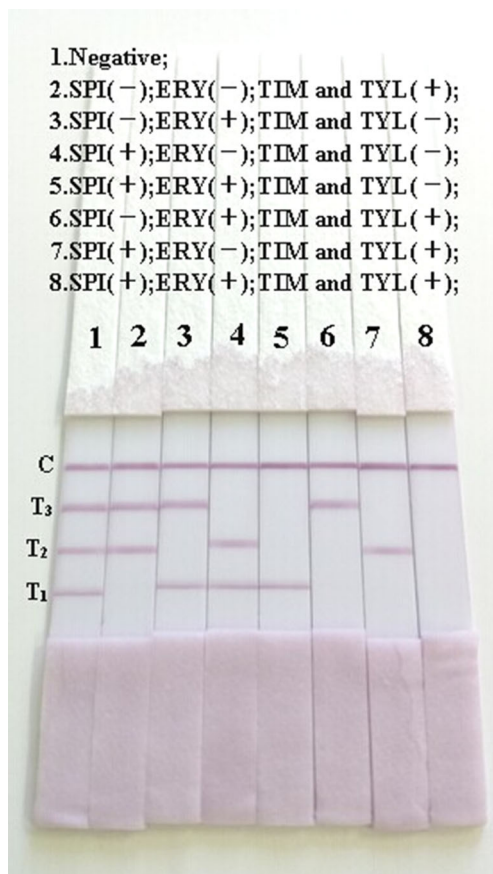
Fig. 2 Schematic description of typical strip test results

Fig. 3 The effect of colloidal gold particle size on sensitivity and color intensity of the test strip; 20, 30, and 40 nm, *from left to right*

Results and Discussion

Optimization of the IGCA Generally, the analytical performance of the IGCA is affected by many factors, such as the colloidal gold particle size, the types of the NC membrane, antigen coated system, etc. This article discussed the following aspects:

A. *Selection of the colloidal gold particles*. In order to evaluate the effect of colloidal gold particle size on sensitivity and color intensity of the test strip, we prepared three diameter sizes of colloidal gold particles by adding 4.5, 6.2, or 8.0 mL of 1 % trisodium citrate into 100 mL of 2.0 % chloroauric acid. The color of colloidal gold solution changed from purple to bright red with the increase of the amount of trisodium citrate. Then, the diameter sizes were determined by a transmission electron microscope and calculated to be 40, 30, and 20 nm, respectively. The experimental results showed that there were no sensitivity differences between the three gold particles. However, the best color intensity and the lowest Abs consumption were obtained by using colloidal gold with a diameter of 40 nm (Fig. 3). This is because the larger the colloidal gold particle size, the more eye-catching of color effect, and the
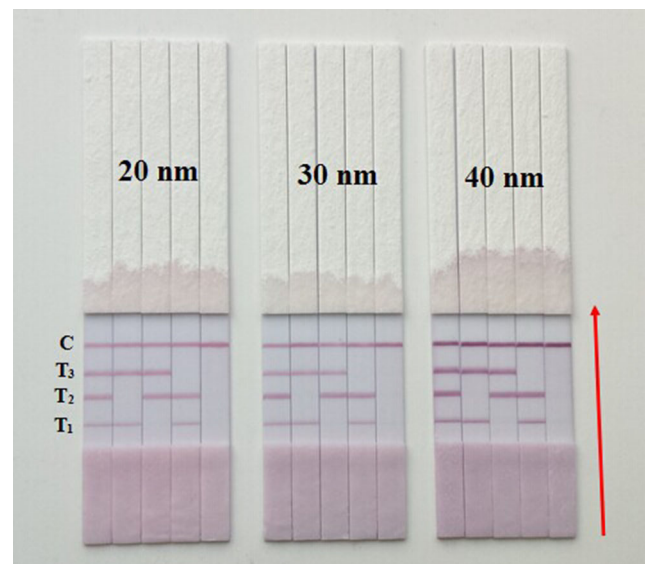
more amount of binding to the Abs. Therefore, the diameter size of 40 nm has been widely used in practise and was selected in our study.

B. *Selection of the NC Membrane*. Since NC membrane is the carrier of test and control lines, but also the occurrence of the immune response, the pore size and capillary migration rate of NC membrane determine the moving speed of the gold-labeled Ab. Three different pore sizes of NC membrane, including Millipore 13520 s, Sartorius CN140, and Mdi 90CNPH, were used to evaluate the sensitivity and color intensity of the test strip. Although there were differences on the moving speeds of gold-labeled Ab, they were able to achieve the desired color intensity and sensitivity within 5 min (Fig. 4). All tested membrane could be used and gives near the same results. The membrane Mdi 90CNPH was used for the following experiments.

C. *Selection of the Coating Buffer and Coating Condition*. Three coating buffers, including carbonate buffer (CB, 0.05 M, pH 9.6), PBS (0.02 M, pH 7.4), and PB (0.02 M, pH 7.4), were evaluated and compared to immobilize three antigens and goat anti-mouse IgG on the NC membrane. The sensitivity and color intensity of the strip test were better using PBS than CB and PB. In addition, the test and control lines would turn yellow after storing for 2 days if the antigens and goat anti-mouse IgG were coated using CB, and the width of test and control lines would spread if they were coated using PB (Fig. 5). This indicated that the salt concentration and pH could impact the immobilization of antigens and goat anti-mouse IgG on NC membrane. Meanwhile, we found that the coating buffer containing a certain concentration of sucrose solution could improve the sensitivity and stability of the test strip, but too high concentration could reduce
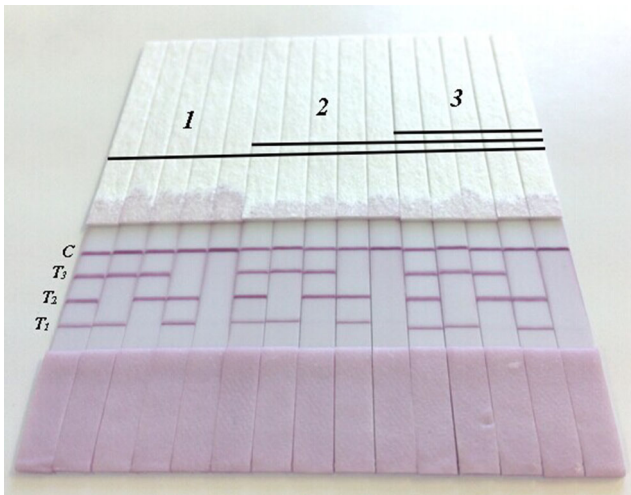
Fig. 4 The effect of NC membrane on sensitivity and color intensity of the test strip. *1*, Mdi 90CNPH; *2*, Millipore 13520 s; *3*, Sartorius CN140

the color intensity of the test strip. Therefore, 0.02 M PBS (2 % sucrose, pH 7.4) was used in this investigation.

The selection of coating time was the same to the previous study (Li et al. 2011). To save time, at 37 °C for 2 h was selected for drying the coated NC membrane, and the NC membrane was not blocked.

D. *Selection of Coated-Position of Antigens*. Since the test strip contains three test lines, and uses the principle of competition, the phenomenon may occur that different coated position of each antigen leads to different sensitivity. To evaluate the effect of coated position of antigens on sensitivity and color intensity of the test strip, three antigens were sprayed onto the NC membrane forming to
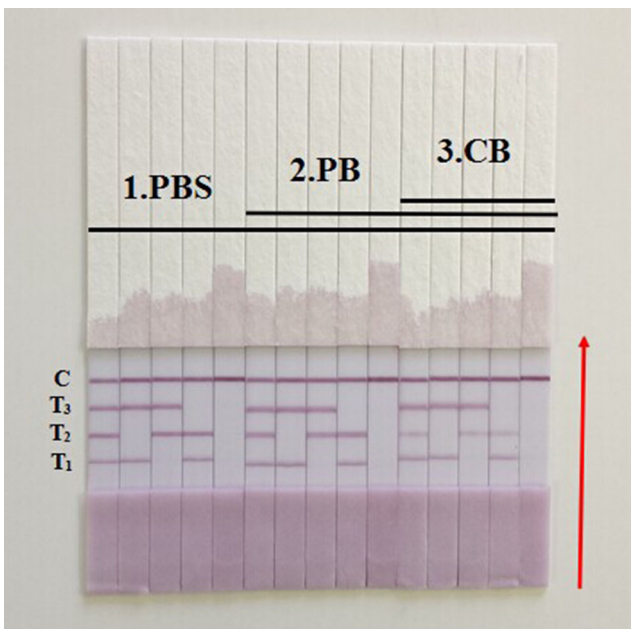

Fig. 5 The effect of coating buffer on sensitivity and color intensity of the test strip. *1*, PBS; *2*, PB; *3*, CB

six kinds of position combinations. The results showed that there were no sensitivity and color intensity differences between the six position combinations (Fig. 6). This may be because the molecular weight of each drug is small, there is no steric hindrance, and the competing reactions are not affected. Therefore, based on personal preferences, TIM-BSA conjugate was sprayed as test line 1, ERY-BSA conjugate was sprayed as test line 2, and SPI-OVA conjugate was sprayed as test line 3, respectively.

E. *Selection of the Resuspended Solution of Gold-Labeled Abs*. It can be said that the stability of gold-labeled Abs directly determine the stability of the test strip; therefore, it is particularly important for the resuspended solution of gold-labeled Abs. The three most commonly used buffer system for the resuspended solution are PB, Tris-HCL, and borate buffer (BB). In addition, the surfactant such as Triton-100 and Tween-20 can facilitate the release of the gold-labeled Abs, while reducing non-specific adsorption of gold-labeled Abs in the NC membrane. Sucrose, BSA, and polyethylene glycol (PEG) are the commonly used protein protective agents which can improve the stability of the gold-labeled Abs (Paek et al. 2000). In our study, the higher sensitivity was obtained by using PB and Triton-100. However, whether surfactant or protein protective agent, too high or too low concentration, is not conducive to the stability and sensitivity of the gold-labeled Abs, therefore, 0.02 M PB (pH 7.4) containing 5 % sucrose, 0.5 % BSA, 0.5 % Triton-100, and 0.03 % sodium azide was selected for the resuspended solution of gold-labeled Abs.

F. *Selection the way of adding sample solution*. The mixed gold-labeled Abs were freeze-dried in the microporous, the test strip was inserted into the sample solution vertically when detection, and the higher sensitivity of the test strip was achieved than the sample solution added levelly. This may be due to the fact that the flow rate of sample solution was relatively slow when the test strip was inserted vertically; more free drugs could combine to the gold-labeled Abs to reduce the competitiveness of coating antigens and to improve the detection sensitivity.

Evaluation of the IGCA

A. *Sensitivity of the IGCA*. The raw milk samples were spiked with different levels of ERY, SPI, TIM, or TYL to determine the detection limit of the strip test. In our work, the detection limit of the strip test was defined as the drug concentration of sample solution which resulted in total invisibility of the test line. The detection limits (LODs) for ERY, SPI, TIM, and TYL were 5, 5, 10, and 20 ng/mL (Fig. 7), respectively, since at those

Fig. 6 The effect of coated position of antigens on sensitivity and color intensity of the test strip
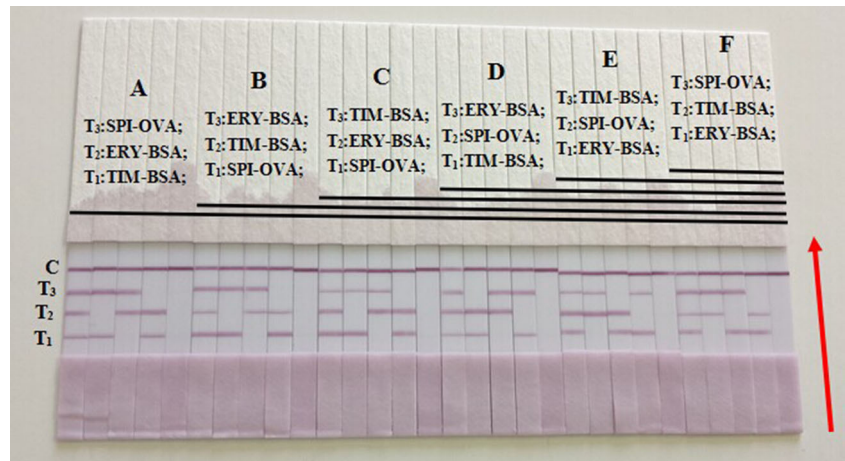
concentration the three test lines will completely disappear. Albrecht et al. reported that the LOD for ERY and SPI in raw milk was 10 and 5.6 ng/mL by an ELISA (Albrecht et al. 1996, 1998); Peng et al. reported the LOD for TYL and TIM in milk were 5.7 and 11.1 ng/mL using an ELISA (Peng et al. 2012); while Luo et al. reported the LOD for TYL and TIM in milk were 25 and 50 ng/mL with an IGCA (Luo et al. 2012). Compared to the above reports, our results obtained showed that the IGCA in our study can detect most kinds of macrolides

Fig. 7 Strip test for the detection limits of SPI, ERY, TIM, or TYL in raw milk

and has the highest sensitivity of multi-component macrolides determination so far (Table 1).

B.	*Specificity of the IGCA*. To evaluate the specificity of the strip, ERY, SPI, TIM, and TYL standard solutions were tested for cross-reactivity by the strip test. The results of cross-reactivity studies demonstrated that the ERY MAb had no cross-reactivity with SPI, TIM, and TYL. The SPI MAb had no cross-reactivity with ERY, TIM, and TYL. The TIM PAb had no cross-reactivity with ERY and SPI, but had 50 % cross-reactivity with TYL; this is because TIM is a semi-synthetic drug of hydrolyzate of TYL, the structures of the two drugs are similar. Many previous reports have shown that TIM or TYL Abs have significant cross-reactivity with TYL or TIM (Peng et al. 2012; Luo et al. 2012; Zhang et al. 2013). This means that the TIM PAb can simultaneously detect TIM and TYL, and the strip test can simultaneously detect ERY, SPI, TIM, and TYL in raw milk, and the LODs fully meet the detection limit requirements of China, the USA, and EU.

C.	*Stability of the IGCA*. The stability of the IGCA was evaluated by accelerated stability test, which the strips
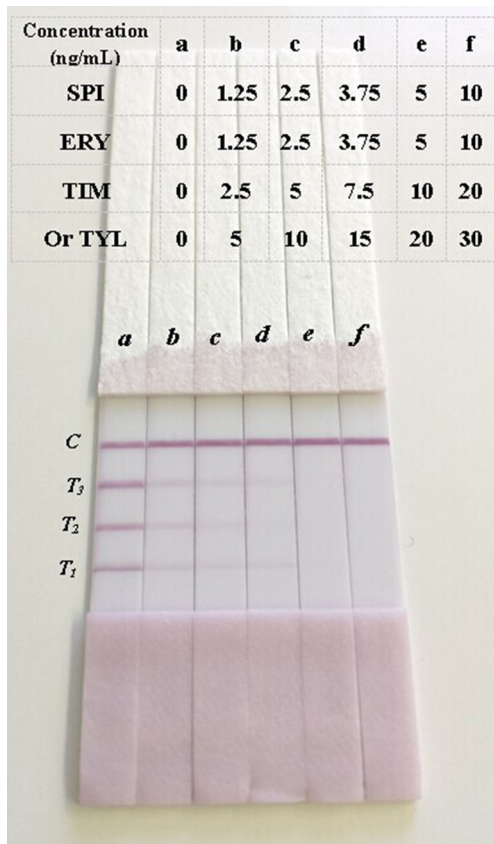
Table 1	Comparison of the LOD for ERY, SPI, TIM, and TYL by the developed IGCA and other immunological methods

| Reference	Method	Target analytes	LOD (ng/mL)
Albrecht et al. (1998)	ELISA	ERY	10
		SPI	5.6
Peng et al. (2012)	ELISA	TIM	11.1
		TYL	5.7
Luo et al. (2012)	IGCA	TIM	50
		TYL	25
_[a]	IGCA	ERY	5
		SPI	5
		TIM	10
		TYL	20

[a] The data obtained in this work

Table 2 Comparison of ERY, SPI, TIM, and TYL analyses using LC–MS/MS and the strip test in some blind raw milk samples

Sample no.	LC–MS/MS (ng/mL)				Strip test[a] (n=3)		
	TIM	TYL	ERY	SPI	T₁	T₂	T₃
1	0	0	0	0	—	—	—
3	0	0	0	19.8	—	—	+++
5	0	0	20.5	20.2	—	+++	+++
6	0	10.5	8.8	0	± ± ±	+++	—
7	4.3	10.5	0	0	+++	—	—
10	20.4	0	16.7	15.8	+++	+++	+++
12	0	0	19.9	0	—	+++	—
14	18.4	0	17.8	19.5	+++	+++	+++
17	0	30.5	0	0	+++	—	—
18	0	0	1.3	0	—	± ± ±	—
19	0	10.3	5.2	0	± ± ±	+++	—
21	0	0	0	2.1	—	—	± ± ±
23	0	29.5	10.3	0	+++	+++	—
24	11.1	0	0	10.2	+++	—	+++
26	0	26.3	20.5	10.3	+++	+++	+++
27	0	0	20.9	1.8	—	+++	± ± ±
30	15.3	0	15.2	14.3	+++	+++	+++
31	5.7	0	8.9	0	± ± ±	+++	—
34	4.3	0	2.0	4.9	± ± ±	± ± ±	+++
35	26.4	0	0	0	+++	—	—
37	0	0	0	37.8	—	—	+++
40	0	27.4	0	35.4	+++	—	+++
43	9.7	0	0	0	+++	—	—
44	0	20.3	0	0	+++	—	—
46	0	0	13.6	0	—	+++	—
48	0	14.8	0	0	± ± ±	—	—
49	0	10.6	3.1	1.8	± ± ±	± ± ±	± ± ±
51	0	0	55.3	48.5	—	+++	+++
54	50.3	0	0	0	+++	—	—
56	10.1	5.4	8.3	10.0	+++	+++	+++
58	0	0	0	2.2	—	—	± ± ±

[a] —, absence of drug; +, presence of drug; ±, weakly positive

Fig. 8 Strip test for the detection of some blind raw milk samples. One sample as a negative control, the others are positive results
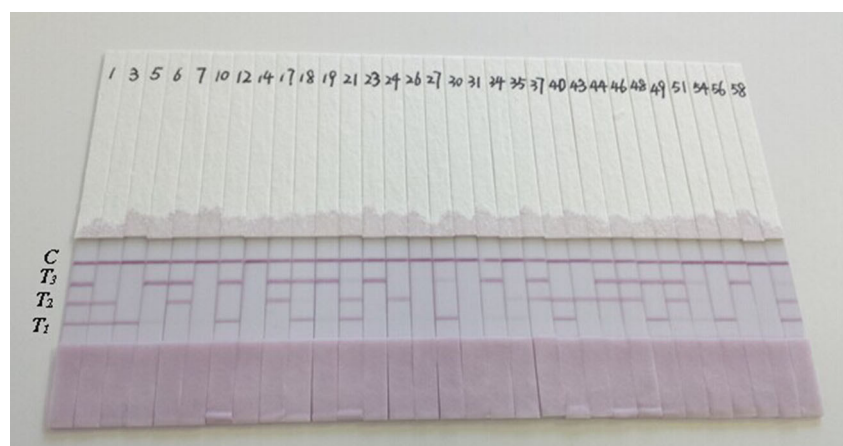
were stored under 45 °C dry condition for 1 month, then the color intensity and sensitivity of the strips were analyzed on 1, 7, 15, 24, and 30 days, respectively. The results showed that the color intensity and sensitivity of the strips remained unchanged after 30 days of accelerated stability test. This indicates that the test strips have good stability and can be stored for 6–12 months at room temperature.

D. *Repeatability and Reproducibility of the IGCA*. The repeatability of the IGCA was measured by five duplicate detection for 20 raw milk samples including positive and negative. The results of five repeat detection for 20 raw milk all showed good consistency, whether the color effect or the inhibitory effect. The reproducibility of the IGCA was evaluated by testing the same samples at different time points. Consistent results were gained at different time points. These results indicated that the IGCA has good repeatability and reproducibility.

Comparative Study Between the Strip Test and LC–MS/MS For quantitation and identification of the four macrolides including ERY, SPI, TIM, and TYL in 60 blind raw milk samples, LC–MS/MS analysis was conducted in parallel with the IGCA as a confirmatory method. The raw milk samples were all analyzed by the IGCA and confirmed by LC–MS/MS. The LOD of the LC–MS/MS method for the four macrolides in raw milk was <0.3 μg/kg. The results showed a good correlation between the two methods (Table 2 and Fig. 8), and false positive or false negative results were not appeared during the entire testing process, indicating that the IGCA we developed had good reliability.

Conclusion

In summary, this is the first report of using an IGCA to simultaneous determine of four macrolides including ERY, SPI, TIM, and TYL in raw milk, and the detection of the four drugs

was completed on a single test strip by three test lines. The samples were detected directly without treatment, and the entire testing process was completed within 10 min. The LOD for ERY, SPI, TIM, and TYL was 5, 5, 10, and 20 ng/mL, respectively. The LODs fully meet the detection limit requirements of China, the USA, and EU, and the sensitivity of the developed method is comparable with the previously published ELISA method. Therefore, the developed strip test is simple, rapid, reliable, sensitivity, and very suitable for simultaneous detecting ERY, SPI, TIM, and TYL residues onsite in large numbers of raw milk. Since this method provides only qualitative and semi-quantitative results, the determined positive samples or suspicious samples should be further confirmed by more sensitive methods such as HPLC, LC–MS, LC–MS/MS, etc.

Conflict of Interest Xiangmei Li has no competing financial interests. Kai Wen has no competing financial interests. Yiqiang Chen has no competing financial interests. Xiaoping Wu has no competing financial interests. Xingyao Pei has no competing financial interests. Qi Wang has no competing financial interests. Ailing Liu has no competing financial interests. Jianzhong Shen has no competing financial interests. Guidelines for the care and use of laboratory animals were followed.

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