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Development of an Immunochromatography Strip for the Rapid Detection of 12 Fluoroquinolones in Chicken Muscle and Liver

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A rapid and sensitive colloidal gold immunochromatography test strip based on one monoclonal antibody with broad-specificity, which can detect 12 fluoroquinolones (FQs), was developed. Antigen and goat anti-mouse IgG were respectively drawn on NC membrane as test line and control line. Gold-labeled antibody was added on a pad and put on one end of the membrane. Fluoroquinolones in sample solution compete with antigen combined on NC membrane for the gold-labeled antibody. When enough fluoroquinolone exists, the test line vanishes as there are no spare gold-labeled antibodies that can bind with antigen on the membrane. The control line always exists when the antibody is activated. The lowest detection limits of the FQs in spiked chicken muscle and chicken liver samples were 25 ng mL^{-1} for norfloxacin and pefloxacin. The lowest detection limit for the other 10 FQs (enrofloxacin, ciprofloxacin, norfloxacin, flumequine, pefloxacin, ofloxacin, lomefloxacin, enoxacin, danofloxacin, amifloxacin, oxolinic acid, and marbofloxacin) was 50 ng mL^{-1}. The whole process involving sample preparation and detection can be finished in <10 min. The results demonstrate that the developed method can be potentially used as a screening tool for the determination of 12 FQ residues in a large amount of samples on site.

KEYWORDS: Fluoroquinolones; residue; colloidal gold; immunochromatography; multiple residue detection

INTRODUCTION

Fluoroquinolones (FQs), a class of broad-spectrum antibiotics that are active against Gram-negative and Gram-positive bacteria, have been widely used in the prevention and treatment of diseases in humans and animals (*1*). Several FQs, for example, enrofloxacin (ENR) and sarafloxacin (NOR), were specifically developed for veterinary use for the treatment of respiratory diseases and gastroenteritis in food-origin animals such as cattle, pigs, and poultry, as well as for diseases in aquaculture.

The wide range of applications and possibility of abuse or misuse of FQs present potential hazards to human health, such as the emergence and spread of drug-resistant bacterial strains and possible induction of cancer (*2*). To protect the health of consumers, maximum residue limits (MRLs) have been established for several FQs by many countries including the European Commission, CAC, and the China Ministry of Agriculture (no. 278.2003.5.22) (*3*).

In the past decade, high-performance liquid chromatography (HPLC) coupled with UV, fluorescence, or mass spectrometry has been utilized commonly for the detection of FQs in animal-origin food (*4–11*); however, these methods are not suitable for screening of a large number of samples due to the complex and time-consuming cleanup procedure and the requirement of sophisticated analytical equipment. It is, therefore, desirable to develop a simple and rapid analytical method for screening FQ residues in animal-derived food.

Recently, enzyme-linked immunosorbent assays (ELISA) have been widely used as a screening tool in environmental and agriculture for trace residue analysis (*12–14*). Compared with HPLC, ELISA needs less time and less sample preparation. An immunogold chromatography method may be an alternative to ELISA as a rapid screening method. It requires the least sample preparation and no equipment. It can analyze many

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samples simultaneously or a single sample with a sensitivity similar to that of ELISA. The technique was first introduced to detect human chorionic gonadotrophin (HCG) in 1980 (*16*). There have been many reports about the detection of small molecules (including agricultural and veterinary drugs) in the environment and animal products (e.g., milk, muscle) over recent years (*15–19*). A direct competitive format has been employed for the detection of small molecules. Antigen is immobilized on NC membrane as capture reagent. The detector reagent, antibody bound to colloidal gold particle, is deposited onto the conjugate pad. Samples are added on a sample pad and allowed to flow along to the other end of the membrane strip. If the sample contains analyte that could be recognized by the antibody, it will compete with the antigen immobilized on the NC membrane to bind with the limited amount of detector reagent. When enough analyte exists, there will be not enough detector reagent to combine with capture reagent, and the signal of the test line will decrease to show a positive result.

The aim of this study was to develop a class-specific immunochromatographic assay to detect the total residues of 12 FQs in chicken muscle and liver samples. As confirmed by the results from ELISA, this immunochromatographic assay has been demonstrated to be rapid, simple, and effective for screening residues in animal-derived food.

MATERIALS AND METHODS

Materials and Chemicals. Enrofloxacin (ENR) (purity, 100%), ciprofloxacin (CIP) hydrochloride (100%), norfloxacin (NOR) (99.6%), flumequine (FLU) (99.5%), and pefloxacin methanesulfonate (PEF) (99.9%) were purchased from the China Institute of Veterinary Drug Control (Beijing, China). Ofloxacin (OFL) (>99.0%), lomefloxacin (LOM) (99.8%), enoxacin (ENO) (>99.0%), danofloxacin (DAN) (>99%), amifloxacin (AMI) (>99%), oxolinic acid (OA) (>99%), and marbofloxacin (MAR) (>99%) were purchased from Sigma Chemical Co. (St. Louis, MO). Ovalbumin (OVA) was also purchased from Sigma Chemical Co. Gold chloride tetrahydrate were supplied by Beijing Regent Corp. (Beijing, China). All other chemicals and solvents were of analytical grade or better and were obtained from Beijing Chemical Reagent Co. Deionized water was prepared using a Milli-Q water purification system (Millipore, Bedford, MA). The nitrocellulose membrane (AE99), the sample pad (Glass 33), and the absorbent pad (CF4) were obtained from Whatman. The UV−vis spectrometer was purchased from Thermo.

Preparation of Capture Agents and Antibodies. The coating antigens were prepared by conjugating NOR to OVA using two methods as described by Wang et al. (*15*).

Two class-specific anti-FQs Mab named C4A9H1 and N2H3A8 were previously prepared (*15*). Antibodies were dialyzed against 0.01 M PBS at 4 °C for 72 h and then dialyzed against 0.005 M NaCl solution for 12 h. The concentration of purified antibody was detected with UV−vis spectrometry and diluted to 100 μg mL^{-1} by deionized water for further use.

Synthesis of Colloidal Gold Particles. Colloidal gold with different diameter sizes was prepared as described by Zhou et al. with some modification (*20*). One hundred milliliters of 0.01% gold chloride tetrahydrate was heated to boiling under stirring, and then 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, or 5 mL of 1% trisodium citrate dehydrate solution was quickly added. After constant heating and stirring for 20 min, the resultant solution was left to cool, and recovered to the initial volume with deionized water, and then scanned with UV−vis spectrometry from 400 to 600 nm to determine the diameter of colloidal gold particles.

Preparation of Colloidal Gold Probe. The pH value of 1 mL of colloidal gold sol was adjusted to 6.5, 7.4, 8.2, and 9.0 by adding 0.1 M K_2CO_3, and then 1 mL of antibody of different concentrations (from 1 to 9 μg mL^{-1}) was added.

The pH value of 10 mL of colloidal gold was adjusted to 7.4 with 0.1 M K_2CO_3, and then 500 μL of purified Mab (100 μg mL^{-1}) was added dropwise. The mixture was shaken and incubated for 10 min, and then 3 mL of 5% BSA was added. After another 10 min of incubation, the solution was centrifuged under 11000*g* for 15 min. Then the gold-labeled antibody (red fluid sediment) was resuspended in dilution buffer for further experimentation.

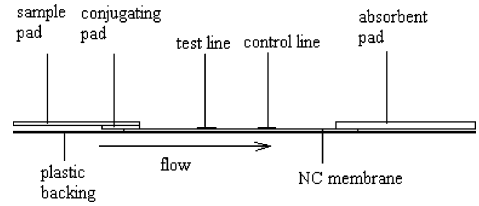

Figure 1. Cross view of a test strip's structure.

Table 1. IC_{50} Values and Cross-Reaction (CR) of Two Antibodies

	C4A9H1		N2H3A8	
FQ	IC_{50} (ng mL^{-1})	CR (%)	IC_{50} (ng mL^{-1})	CR (%)
CIP	8.9	100	3.6	58
ENR	10.9	87	3.4	62
NOR	12.5	71	2.1	100
OFL	16.5	54	3.3	63
DAN	16.5	54	3.4	62
PEF	18	49	2.2	100
AMI	17.5	51	3.3	63
LOM	22	40	4.4	48
ENO	20	45	3.3	63
FLU	25.5	35	3.9	54
OA	22	40	3.3	63
MAR	21.5	41	3.7	57
DIF	284	3.1	909	<0.01
SAR	307	2.9	959	<0.01

Table 2. Colors and Diameter Sizes of Colloidal Gold Prepared with Different Amounts of Sodium Citrate

vol (mL) of sodium citrate added in 100 mL of 0.01% gold chloride	color of colloidal gold	diameter size (nm) of gold sol
1.0	dark red	40
1.6	red	20
2.5	salmon pink	15

Immobilization of Capturing Reagent. NOR−OVA and goat anti-mouse IgG were separately diluted to 100 μg mL^{-1} with coating buffer (0.05 M carbonate buffer, pH 9.6) and then dispensed onto the NC membrane with pens as test line and control line. The prepared NC membrane was dried at 37 °C overnight.

Assembly of Testing Strip. The assembly procedure was similar to that described by Zhou et al. (*19*). As shown in **Figure 1**, the test strip consists of NC membrane, conjugate pad, sample pad, and absorbent pad. All of these parts were pasted on a backing plate. The NC membrane was pasted on the center of the backing plate. The

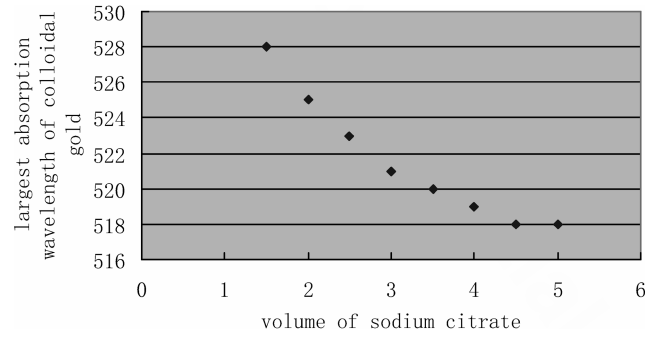

Figure 2. Largest absorption wavelength of colloidal gold prepared with different amounts of sodium citrate.

Table 3. Detection Results of Standard Solution (I, Negative Result, both Control Line and Test Line Appeared Clearly; II, Weak Negative Result, the Color of the Test Line Was Lighter than That of Negative Results but Still Visible; II, Weak Positive Result, the Test Line Was Weakened Obviously but Did Not Vanish Completely; IV, Positive Result, Test Line Vanished)

FQ	concentration of standard solution					LOD	CR%
	0 ng mL^{-1}	2 ng mL^{-1}	5 ng mL^{-1}	10 ng mL^{-1}	20 ng mL^{-1}		
CIP	I I I	II II II	III III III	IV IV IV	IV IV IV	10	50
ENR	I I I	II II II	III III III	IV IV IV	IV IV IV	10	50
NOR	I I I	III III III	IV IV IV	IV IV IV	IV IV IV	5	100
OFL	I I I	II II II	III III III	IV IV IV	IV IV IV	10	50
DAN	I I I	II II II	III III III	IV IV IV	IV IV IV	10	50
PEF	I I I	III III III	IV IV IV	IV IV IV	IV IV IV	5	100
AMI	I I I	II II II	III III III	IV IV IV	IV IV IV	10	50
LOM	I I I	II II II	III III III	IV IV IV	IV IV IV	10	50
ENO	I I I	II II II	III III III	IV IV IV	IV IV IV	10	50
FLU	I I I	II II II	III III III	IV IV IV	IV IV IV	10	50
OA	I I I	II II II	III III III	IV IV IV	IV IV IV	10	50
MAR	I I I	II II II	III III III	IV IV IV	IV IV IV	10	50

conjugating pad (glass fiber) on which the diluted Au-Ab was dispensed previously was pasted on the plate by overlapping 2 mm with the NC membrane. The sample pad was pasted on the same end by its margin justified to the conjugating pad. The absorbent pad (CF4) was pasted on the other end of the NC membrane by the same overlapping of 2 mm. Then the whole assembled plate was cut into 4 mm strips and stored under dry conditions at room temperature.

Detection of Standard Solution. Standard solutions of different concentrations were dissolved with 0.01 M PBS (pH 7.4), and then the detection limit was determined. The blank sample was the dilution buffer (0.01 M PBS). Sixty microliters of the solution was added dropwise onto the sample pad and allowed to migrate upward, and the result was judged after 5 min. Two red lines displayed on the T and C regions were judged as negative results. Positive result was judged by the complete disappearance of the test line. When the color of the test line was obviously weaker than that of the control line, it was defined as a weak positive result. The control line always exists when a test strip was effectual.

Sample Pretreatment. Prior to running the immunochromatographic assay, chicken muscle or liver was homogenized, and then 1 g of tissue sample was weighed into a 50 mL polypropylene centrifuge tube. Five milliliters of 0.02 M PB (pH 7.4) was mixed with tissue sample for about 5 min. Then 80 μL of the sample extract was pipetted onto the sample pad of the strip for analysis.

Analysis of Spiked Samples. One gram of homogenized sample was accurately weighed into a 50 mL polypropylene centrifuge tube. Standard solutions (1000 ng/mL, prepared in 0.02 M PBS) were added into tissue samples to produce spiked concentrations of 0, 10, 25, 50, and 100 μg/kg. Then the samples were stirred for 10 min and kept still for another 10 min before they were used. Blank and spiked samples were detected with an immunochromatography strip ($n = 5$).

Analysis of Real Samples. Some real samples are known as blank samples, and some of them come from chicken feed by enrofloxacin. Twenty chicken muscle samples and 20 chicken liver samples were analyzed using one-step strips. Then the results were confirmed by ELISA.

RESULTS AND DISCUSSION

Antibody Preparation and Characterization. Two antibodies against multiple FQs were prepared in our laboratory. The IC_{50} and cross-reactivity values of the two antibodies are indicated in **Table 1**. The antibody C4A9H1 showed the broadest cross-reactivity with 14 FQs, but its IC_{50} values for SAR and DIF were about 300 ng/mL (*15*), which could not meet the sensitivity requirement for residue detection. Although another antibody, N2H3A8, has no cross-reactivity with SAR and DIF, its IC_{50} values for the other 12 drugs (<4.5 ng mL^{-1}) were lower than that of C4A9H1; thus, N2H3A8 was finally chosen for the following experiment. As discussed in Wang et al.'s paper (*15*), the existence of the fluorophenyl group at position 1 of ring B blocked SAR and DIF from binding with the antibody. In this respect, SAR and DIF cannot be diagnosed by the strip. In further research, antibody that can detect SAR and DIF should be added into the detection system to complete the assay. Using a mouse monoclonal antibody isotyping kit, N2H3A8 was determined to be IgG1 isotype with kappa light chain.

Preparation of Colloidal Gold. The preparation of colloidal gold is a reduction reaction using sodium citrate as reducing agent. On the condition of fixed reaction temperature and reaction time, there is a correlation between the diameter sizes of colloidal gold particles and the amount of sodium citrate. Different diameter sizes of colloidal gold particles make the solution appear different colors (*20*).

As shown in **Table 2**, the color of colloidal gold changed from dark red to pink and then to reddish as the amount of sodium citrate in the reaction system increased. This result was identical to that reported by Zhou et al. (*20*). In addition, the maximum absorption wavelength of colloidal gold declined from 530 to 518 nm when the diameter of the colloidal gold particle decreased (**Figure 2**). However, we observed that when the amount of sodium citrate increased to >2.5 mL, the change of diameter of gold particles was not obvious, and when it increased to 5 mL, the diameter of gold particles did not decline any more.

Optimization of Immunochromatographic Strip. As a preliminary study, several sizes of colloidal gold particles were tested for conjugation with antibody. G40 (40 nm gold particle) was generally used in the production of colloidal gold probe because it can lead to better color signal (*20, 24, 25*). Additionally, G25 was also chosen in some research (*24, 26*). In this study, it was found that smaller sizes of gold particle had better stability in solution than larger gold particles after conjugation with antibody; thus, 15 nm colloidal gold was used for further experimentation.

Previous studies had shown that pH value and antibody amount are important for gold−Ab conjugation; many researchers supported that conjugation should be conducted under a pH value slightly higher than the p*I* of the antibody due to the maximal antibody absorption at this pH. This optimal pH value can be determined by measuring the differential absorbance according to the method described by Cho and Paek (*22*). In contrast, we utilized a two-dimensional titration method to determine the optimal pH value and antibody amount. The pH values (6.0, 7.4, 8.2, and 9.0) and antibody amounts (from 1 to 9 μg per milliliter of colloidal gold) were screened for the optimum combination to obtain the best sensitivity of the strip. As a result of titration, the optimal pH value was found to be 7.0 and the optimum amount of antibody was 1 μg for 1 mL of colloidal gold.

Subsequently, various coating conditions of OVA−NOR and goat anti-mouse IgG immobilized on NC membrane were compared. Among three coating buffers (CB, PB, PBS), carbonate buffer (CB) gives the best sensitivity and higher signal, which indicates that a higher pH value was of more benefit for protein absorption on NC membrane. Because the study of Zhou et al. (*20*) revealed that the best result was obtained using PBS as coating buffer, it appears that different coating antigens may need different conditions to bind on NC membrane.

Table 4. Detection Results of Blank and Spiked Samples (See **Table 3** for Interpretation of Symbols)

		spiked concentrations				
FQ	sample	0 μg kg^{-1}	10 μg kg^{-1}	25 μg kg^{-1}	50 μg kg^{-1}	100 μg kg^{-1}
CIP	chicken muscle	I I I I I	II II II II II	III III III III III	IV IV IV IV IV	IV IV IV IV IV
	chicken liver	I I I I I	II II II II II	III III III III III	IV IV IV IV IV	IV IV IV IV IV
ENR	chicken muscle	I I I I I	II II II II II	III III III III III	IV IV IV IV IV	IV IV IV IV IV
	chicken liver	I I I I I	II II II II II	III III III III III	IV IV IV IV IV	IV IV IV IV IV
NOR	chicken muscle	I I I I I	III III III III III	IV IV IV IV IV	IV IV IV IV IV	IV IV IV IV IV
	chicken liver	I I I I I	III III III III III	IV IV IV IV IV	IV IV IV IV IV	IV IV IV IV IV
OFL	chicken muscle	I I I I I	II II II II II	III III III III III	IV IV IV IV IV	IV IV IV IV IV
	chicken liver	I I I I I	II II II II II	III III III III III	IV IV IV IV IV	IV IV IV IV IV
DAN	chicken muscle	I I I I I	II II II II II	III III III III III	IV IV IV IV IV	IV IV IV IV IV
	chicken liver	I I I I I	II II II II II	III III III III III	IV IV IV IV IV	IV IV IV IV IV
PEF	chicken muscle	I I I I I	III III III III III	IV IV IV IV IV	IV IV IV IV IV	IV IV IV IV IV
	chicken liver	I I I I I	III III III III III	IV IV IV IV IV	IV IV IV IV IV	IV IV IV IV IV
AMI	chicken muscle	I I I I I	II II II II II	III III III III III	IV IV IV IV IV	IV IV IV IV IV
	chicken liver	I I I I I	II II II II II	III III III III III	IV IV IV IV IV	IV IV IV IV IV
LOM	chicken muscle	I I I I I	II II II II II	III III III III III	IV IV IV IV IV	IV IV IV IV IV
	chicken liver	I I I I I	II II II II II	III III III III III	IV IV IV IV IV	IV IV IV IV IV
ENO	chicken muscle	I I I I I	II II II II II	III III III III III	IV IV IV IV IV	IV IV IV IV IV
	chicken liver	I I I I I	II II II II II	III III III III III	IV IV IV IV IV	IV IV IV IV IV
FLU	chicken muscle	I I I I I	II II II II II	III III III III III	IV IV IV IV IV	IV IV IV IV IV
	chicken liver	I I I I I	II II II II II	III III III III III	IV IV IV IV IV	IV IV IV IV IV
OA	chicken muscle	I I I I I	II II II II II	III III III III III	IV IV IV IV IV	IV IV IV IV IV
	chicken liver	I I I I I	II II II II II	III III III III III	IV IV IV IV IV	IV IV IV IV IV
MAR	chicken muscle	I I I I I	II II II II II	III III III III III	IV IV IV IV IV	IV IV IV IV IV
	chicken liver	I I I I I	II II II II II	III III III III III	IV IV IV IV IV	IV IV IV IV IV

The time of drying was then tested from 15 min to 12 h. When the drying time increased, the color signal of negative sample seems to be more intense; therefore, overnight at 37 °C was chosen as the drying condition. The NC membrane was blocked after coating. Compared with the membrane unblocked, blocked membrane allows both sample solution and gold-labeled antibody to flow more rapidly and demonstrates clearer background. However, improper blocking method could result in decreased signal strength and may be harmful to the sensitivity of the test strip. The effect of the three blocking buffers (0.1% decreamed milk, 0.01% BSA, and 0.1% casein) was evaluated. As the best sensitivity was obtained when the membrane was blocked with 0.01% BSA, it was selected as blocking buffer for further experiments.

The effect of dilution buffer for detection reagent was then investigated. First, the influence of surfactant (Tween 20 and Triton 100) on assay performance was evaluated. The strength of signal decreased as the concentration of surface active agent increased. Both Tween 20 and Triton 100 can make detection reagent and sample solution flow favorably at the concentration of 0.3%, but better sensitivity was obtained when Tween 20 was contained in the dilution buffer; hence, 0.3% Tween 20 were chosen as final dilution buffer. BSA and PEG are both utilized in dilution buffer of Au−Ab as protection reagents of antibody. By trial and error, we found that the best sensitivity and stability for the strips were obtained when 0.2% BSA and 0.4% PEG were included in the dilution buffer. Finally, we evaluated the effect of sucrose on the strip sensitivity and stability. The addition of sucrose in the dilution buffer made the Au−Ab on the conjugation pad more stable. As a lower concentration of sucrose may lose the ability to protect Au−Ab and a 5% concentration could slightly reduce the sensitivity of the strip, 3% of sucrose was finally selected.

Limits of Detection. Standard solutions of 12 drugs are detected by the optimal test strip to find the lowest detection limit and the cross-reactivity. For different drugs, the strips have lowest detection limits from 5 to10 ng/mL. The results are shown in **Table 3**. We can see that the sensitivity of the immunogold strip is limited by the sensitivity of antibody used to bind with gold particle and has a similar cross-reactivity with it. After optimization, the lowest detection limit of the immunogold test strip is slightly higher than the level of the detection limit of ELISA processed with the same antibody.

Analysis of Spiked Samples. The greatest merits of the immunochromatographic assay, its simplicity and speed, cannot be demonstrated without the simplest sample preparation. Therefore, in all of our experiments, samples are directly extracted with PBS and added to the test strip. The detection results are shown in **Table 4**. All of the test lines declined obviously at the spike level of 25 ng mL^{-1}, and many strips indicated positive results. To avoid false-negative results, 25 ng mL^{-1} for NOR and PEF and 50 ng mL^{-1} for other FQs were determined as suitable limits of detection.

Analysis of Real Samples. Twenty chicken muscle samples and 20 chicken liver samples were analyzed using one-step strips and then confirmed with ELISA. Three muscle samples and two liver samples were judged to be positive. As the lowest detection limit of the gold chromatography assay is >25 μg/kg for each sample, drug concentrations lower than these limits cannot be

Table 5. Detection Results of Real Samples (See **Table 3** for Intepretation of Symbols)

muscle		liver	
result of strip	result of ELISA[a] (total residue of fluoroquinolones, μg kg^{-1})	result of strip	result of ELISA (μg kg^{-1})
I	1.5	I	2.0
I	2.0	I	2.5
I	2.2	I	2.4I
I	1.9	I	2.8
I	2.3	I	2.3
IV	81.5	I	2.4
I	4.4	I	2.6
I	2.5	I	2.3
I	1.8	I	2.5
IV	67.7	I	2.1
I	3.7	I	6.4
IV	79.2	I	2.3
I	1.8	I	2.1
I	1.6	I	2.5
I	7.5	I	2.4
I	1.4	IV	93.5
I	8.7	IV	72.4
I	2.3	I	2.6
I	2.5	I	2.5
I	1.9	I	2.1

[a] The ELISA kit for total residue detection of FQs used an anticiprofloxacin IgG as detector reagent, so the total residue of fluoroquinolones is shown as the amount of cipfloxacin. The lowest detection limits of the ELISA kit are 3.0 μg/kg for chicken muscle and 3.5 μg/kg for chicken liver. Results data lower than the detection limits should be considered as negative results.

recognized. The confirmation step with ELISA also give the same results. The detection results are shown in **Table 5**. This result shows that the two methods corresponded well, and the immunochromatographic strip gave neither false-positive nor false-negative results.

In this study a gold immunochromatography assay was developed to detect 12 fluoroquinolone residues in chicken muscle and liver. The detection limits meet the requirement of CAC, and the whole detection process can be finished within 5 min; thus, the developed immunostrip is suitable for detecting fluoroquinolone residues in large numbers of chicken muscle and liver samples on site. As this method provides only preliminary qualitative results, the determined positive sample should be further confirmed by more accurate and quantitive methods such as HPLC and LC-MS/MS.

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