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Development of a Lateral Flow Colloidal Gold Immunoassay Strip for the Rapid Detection of Olaquindox Residues

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ABSTRACT: A rapid immunochromatographic lateral flow test strip of competitive format has been developed for the specific determination of olaquindox (OLA) residues in pig urine and muscle tissues. The sensitivity of the test strip was found to be 1.58 ± 0.27 μg/kg and 1.70 ± 0.26 μg/kg of OLA in pig urine and muscle tissues, and the lower detection limit was 0.27 ± 0.08 μg/kg and 0.31 ± 0.07 μg/kg respectively. For negative pig urine and muscle samples spiked with 4, 12, and 36 μg/kg, the recovery range was 83.0−94.0% and 78.8−87.4% and the coefficient of variation scope [CV (%)] was 3.17−7.41% and 4.66−7.64% respectively. Parallel analysis of OLA samples from pig urine and muscle tissue showed comparable results from the test strip and HPLC. Each test requires 5−8 min, and the test strip can provide a useful screening method for quantitative, semiquantitative, or qualitative detection of OLA residues.

KEYWORDS: olaquindox, test strip, colloidal gold, immunoassay, rapid test

INTRODUCTION

Olaquindox (OLA, *N*-(2-hydroxyethyl)-3-methyl-2-quinoxaline carboxamide-1,4-di-*N*-oxide) is one of the quinoxaline *N*,*N*-dioxides that are widely used to promote growth, improve feed efficiency, and control both swine dysentery and bacterial enteritis in pig production.[1] It is active against coliform bacteria, where it inhibits DNA synthesis.[2] Olaquindox is genotoxic and possibly a germ cell mutagen that has been shown to be mutagenic in a variety of test systems in vivo and in vitro.[3−6] Therefore, potentially toxic residues of OLA in edible animal-origin product could affect human health.[7] Studies show that occupational exposure to olaquindox can cause allergic and photoallergic contact dermatitis.[8,9] The European Union has banned the use of OLA since 1999. The Ministry of Agriculture of China stipulated that OLA can only be used for pigs weighing less than 35 kg with a withdrawal period of 35 days. The maximum residue limit (MRL) for olaquindox has been set at 4 μg/kg for muscle tissue.

So far, classical analytical methods have been described for the detection of OLA in tissues and feed, including high performance liquid chromatography (HPLC),[10−12] liquid chromatography−mass spectrometry (LC−MS),[13] and LC−MS/MS.[14−16] These methods require extensive sample preparation as well as highly trained individuals to operate sophisticated instruments and interpret complicated chromatograms or spectral results. Consequently, these traditional methods, although highly accurate, are time-consuming, costly, and generally not suitable for screening large amounts of samples and real-time detection.[17] In addition, enzyme-linked immunosorbent assay (ELISA) is also used for detect of OLA, but the sensitivity cannot meet the national standard.[18]

Therefore, to detect the presence of OLA under working conditions, rapid and inexpensive screening methods are required. Recently immunochromatographic lateral flow test strips have become a popular diagnostic tool for detecting high molecular mass analytes such as viruses,[19,20] bacteria,[21] hormones,[22] and parasite antigens[23,24] with the sandwich format.[25] For the smaller analytes the competitive format lateral flow assay can be used.[26,27] However, lateral flow devices or even other immunoassays for screening OLA have not been reported. In this study, we produced a panel of monoclonal antibodies (mAb) specific for OLA and developed a lateral flow immunoassay strip with the mAb, which was shown to be suitable for the rapid detection of OLA residues in pig urine and muscle tissues.

MATERIALS AND METHODS

Chemicals and Materials. Olaquindox, carbadox, maquindox, sulfadiazine, chloramphenicol, oxolinic acid, neomycin, tetracycline, penicillin, ractopamine, sulfamonomethoxine, and a mouse monoclonal antibody isotyping kit were purchased from Sigma (St. Louis, MO, USA); bovine serum albumin (BSA) and ovalbumin (OVA) were bought from BDH (VWR International Ltd.); goat anti-mouse IgG antibody (whole molecule) was obtained from Sino-American Biotechnology Co. (Luoyang, China); FCA, FIA, and EDC were purchased from Pierce; PEG1500 was from Roche (Mannheim, Germany); 96-well culture plates were bought from Nunc; RPMI-1640, HAT, and HT medium were purchased from Invitrogen; nitrocellulose membrane, glass fiber, and absorbent pad were purchased from Millipore; eight-week-old female BALB/c mice were obtained from the Laboratory Animal Center, Zhengzhou University, China, and raised under strictly

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controlled conditions; other reagents and solvents were of analytical grade or higher. Double deionized water (DDW) was made in the Key Laboratory of Animal Immunology of the Ministry of Agriculture.

The assay buffer consisted of 0.01 mol/L phosphate-buffered saline (PBS) (pH 7.4), containing 145 mmol/L NaCl. The washing buffer (PBST) consisted of assay buffer containing 0.1% (v/v) Tween-20.

Apparatus. Microplate Readers 450/550 were from Bio-Rad (Richmond, CA). Milli-Q water was obtained from Millipore (Bedford, MA). An XYZ Biostrip Dispenser, CM 4000 Cutter, and TSR3000 membrane strip reader were purchased from Bio-Dot. Ultrasonic cell disrupter system was from Ningbo Scientz Biotechnology Co., Ltd.

Preparation of Immunogens and Coating Antigens. The derivative of olaquindox, called OLA-HS, was synthesized by the succinic anhydride method.[28] Briefly, 26.30 g of OLA and 13.00 g of succinic anhydride were dissolved in dimethyl sulfoxide (DMSO) and heated at 90 °C for 1 h. The solution was combined with 400 mL of DDW after being cooled to room temperature (RT). While stirring, a large amount of yellow precipitate was separated out. The precipitation was filtrated, washed and recrystallized three times, and then dried for conjugation.

OLA−BSA and OLA−OVA conjugates were made by an active ester method.[29] Briefly, 10.96 mg of OLA-HS, 34.00 mg of *N*,*N*-dicyclohexyl carbodiimide (DCC) and 4.17 mg of *N*-hydroxysuccinimide (NHS) were dissolved in 700 μL of *N*,*N*-dimethylformamide (DMF) and stirred for 8 h at RT. 20.00 mg of BSA dissolved in 2.00 mL of 0.01 mol/L, pH 7.4 PBS was added to the above solution and stirred overnight at 4 °C. The conjugates were purified by dialysis against 2 L of 0.01 M PBS (pH 7.2) for nine changes to remove the uncoupled free hapten and then lyophilized. The conjugation ratio was calculated through UV scanning. According to derivation of Lambert−Beer law, the following formula can be used to calculate the conjugation ratio.

$$C_a/C_b = (AC_{am} \cdot KB_{bm} - AC_{bm} \cdot KB_{am})/(AC_{bm} \cdot KA_{am} - AC_{am} \cdot KA_{bm})$$

C_a/C_b stand for the molar ratio of A and B (antigen and carrier) in the conjugate. AC_{am} and AC_{bm} stand for optical density of the conjugate at the maximum absorption wavelength of A and B. KA_{am} and KB_{bm} represent the molar extinction coefficient of A and B at their maximum absorption wavelength. KA_{bm} represents the molar extinction coefficient of A at maximum absorption wavelength of B. KB_{am} stands for the molar extinction coefficient of B at maximum absorption wavelength of A.

Production of mAb against OLA. *Immunization of Mice.* Three 8-week-old BALB/c female mice were immunized with OLA−BSA conjugates. The first dose consisted of 50 μg of immunogen for injection subcutaneously as an emulsion of PBS and Freund's complete adjuvant. Three subsequent injections were given at 3-week intervals with the same dosage of immunogen emulsified in Freund's incomplete adjuvant. Antisera were collected 4 weeks after the fourth immunization and were screened for anti-OLA activity by ciELISA. The mouse with the highest anti-OLA activity received a fifth injection intraperitoneally. Three days later, the spleen of the injected mouse was removed for hybridoma production.

Cell Fusion and Hybridoma Screening. Hybridomas secreting anti-OLA antibodies were generated by standard methods.[30,31] Briefly, the spleen of the immunized mouse was removed, and the splenocytes were isolated and fused with NS0 cells using PEG1500. The fused cells were then distributed into 96-well culture plates, in which mouse peritoneal macrophages were prepared on the day before the fusion and were grown with the selective HAT medium. Ten days after fusion, supernatants of hybridoma colonies were recovered and screened by indirect ELISA for secretion of mAb binding to OLA. Selected clones were subcloned by limiting dilution. Ascites fluids were produced in paraffin-primed BALB/c mice. The subclass of the isotypes of the antibody was determined by using a mouse monoclonal antibody isotyping kit. Measurement of monoclonal antibody affinity (Ka) was carried out according to the procedure described by Batty et al.[32] That is a classical method called noncompetitive enzyme immunoassay. According to the following formula, Ka can be calculated.

$$\mathrm{Ka} = (n-1)2(n[\mathrm{Ab}']t - [\mathrm{Ab}]t)$$
$$n = [\mathrm{Ag}]t/[\mathrm{Ag}']t$$

$[\mathrm{Ag}]t$ and $[\mathrm{Ag}']t$ stand for two different concentrations of coating antigen, and $[\mathrm{Ab}]t$ and $[\mathrm{Ab}']t$ represent the corresponding concentrations of the mAb.

Preparation of Colloidal Gold Labeled mAb. Colloidal gold with a mean diameter of 15 nm was produced by reduction of gold chloride with 1% sodium citrate. Briefly, 50 mL of 0.01% gold chloride trihydrate solution in Milli-Q water was heated to boiling, and then 1.5 mL of 1% sodium citrate solution was added while stirring. After the color changed, the solution was boiled for another 5 min to complete the reduction of the gold chloride, and then cooled to and stored at RT with 0.05% sodium azide added. When preparing the colloidal gold labeled mAb, colloidal gold solution was adjusted to pH 9.0 with 0.2 mol/L sodium carbonate. The optimum protein concentration for labeling was determined by the following steps: 25 μL of anti-OLA mAb solution was 2-fold serially diluted in DDW, and then 25 μL of colloidal gold solution was added. Mixtures were then incubated for 15 min at RT, and 100 μL of 10% NaCl solution was added. The color of the samples changed from brilliant red to blue as the concentration of mAb decreased. The optimum concentration of mAb for colloidal gold labeling was the lowest concentration of mAb solution that did not change color.

Two milliliters of mAb solution, at the optimum concentration of 2 μg/mL, was incubated with 10 mL of colloidal gold solution (pH 9.0) for 30 min at RT. After the addition of 1 mL of 10% BSA solution in 20 mmol/L sodium borate (pH 9.0), the mixture was incubated at RT for another 10 min, and the labeled mAb was then washed using repeated centrifugation (25000*g*) at 10 °C for 30 min with 20 mmol/L sodium borate (pH 9.0) containing 1% BSA and 0.1% sodium azide. The precipitate was then resuspended in the PBST and stored at 4 °C for use.

Preparation of the Conjugate Pad. Conjugate solution was prepared by diluting the colloidal gold labeled mAb to OLA with 20 mmol/L sodium borate buffer (pH 8.0) containing 8.75% (w/v) sucrose, 8.75% (w/v) BSA, 0.6 mol/L NaCl, 10 mmol/L EDTA, and 0.1% (w/v) NaN_3 to a final concentration of 2 μg/mL. A conjugate pad was made by dipping a 7 × 300 mm glass fiber (Millipore) in the conjugate solution and then drying for 1 h at 56 °C. The pad was then stored in a desiccator at RT.

Immobilization of Capture Reagents. The OLA−BSA (1 mg/mL) and goat anti-mouse IgG (1 mg/mL) were applied to the nitrocellulose membrane as the test and control lines, respectively. The test and control lines were situated 0.5 cm apart at the center of the membrane. These reagents were applied in the form of dots at 50 dots mL^{-1} cm^{-1} on the membrane. After drying for 1 h at 40 °C, the membrane was blocked with 2% (w/v) BSA and then dried, sealed, and stored under dry conditions.

Preparation of Sample Pad and Absorbent Pad. Sample and absorbent pads of C048 (Millipore) were made from nonwoven, 100% pure cellulose fiber. The sample pad was cut to 15 × 300 mm and saturated with a buffer (pH 8.0) containing 20 mmol/L sodium borate, 2.0% (w/v) sucrose, 2.0% (w/v) BSA, and 0.1% (w/v) NaN_3 and then dried and stored as described above. The absorbent pad was cut to 40 × 300 mm.

Assembly of the Strip. The sample pad, conjugate pad, blotted membrane, and absorption pad were assembled on the plastic backing support board sequentially with a 1−2 mm overlap and covered by color film at both ends. The master card was cut to 3 mm width strips using a CM 4000 Cutter (Bio-Dot). Strips were then sealed in a plastic bag in the form of desiccant gel and stored at 4 °C.

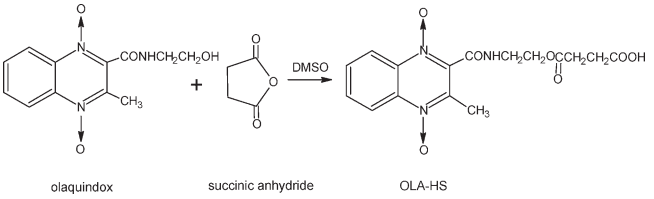

Figure 1. Chemical structures of the synthetic routes to OLA-HS by the succinic anhydride method.

Test Procedure and Principle. Eighty microliters of standard solution or sample extract was added onto the sample pad, and the solution migrated toward the absorbent pad within 5 min. When OLA was absent from the sample, all of the detection reagent would be trapped by capture reagent to form an easily visible test line. When OLA is present in the sample, it competes with the immobilized capture reagent for the limited amount of detection reagent. The more OLA in the sample, the weaker the test line color. If sufficient OLA is present in the sample, it will completely block the reaction with the capture reagent; thus, there is no visible test line on the nitrocellulose membrane. Provided that the test strip and the test procedure are correct, the control line is always visible. If no colored capture line or only a red color at the test line appears, the strip was invalid, and the test should be repeated using a new strip.

Preparation of Spiked Pig Urine and Muscle Samples. A standard stock of OLA was prepared by diluting the initial solution prepared in DDW to give a final stock solution at 1 mg/mL. The stock solution was serially diluted with PBS to give the working standard solutions (0, 1, 2, 4, 8, 16, 32 ng/mL) to be used for spiking 1 mL of negative pig urine and 1 g of negative normal minced pig muscle extracts.

Sample Pretreatment for Strip Test. Briefly, the pig urine samples were centrifuged at 4000*g* for 10 min at RT and the supernatants were collected for detection. One gram of normal minced pig muscles was homogenized with 1 mL of PBS (pH 7.4) by ultrasonic cell disrupter system. The homogenates were mixed on a vortex mixer for 10 min and then centrifuged at 3000*g* for 10 min at RT. The supernatants were analyzed either directly or following further dilution as required by the strip test.

Evaluation of the Test Strip Performance. The sensitivity of the test strips was determined by testing a series of diluted OLA standard sample extracts, in which various concentrations of OLA at 0, 1, 2, 4, 8, 16, and 32 ng/mL in DDW were added . The assays were carried out in triplicate by using the test strip methods as described above. The relative optical density (ROD) of the test line was measured with a TSR3000 membrane strip reader (Bio-Dot). The standard curve was constructed by plotting the G/D-area-ROD or G/peak-ROD values obtained from the standard samples against its logarithm concentrations. G/D-area-ROD means density value of the sampled line points, multiplied by the area of the sampling window on the image. G/peak-ROD means maximum density value of sampled line points. The IC_{50} and the lower detection limit (LDL) were calculated from the regression equation. The linearity of the analytes was assessed by the coefficient of determination (R^2).

To identify the specificity of the test strip the sample of OLA and competitors were spiked in pig urine samples. The competitors carbadox, maquindox, sulfadiazine, chloramphenicol, oxolinic acid, neomycin, tetracycline, penicillin, ractopamine, and sulfamonomethoxine were spiked at a concentration of 2000 ng/mL, respectively.

Intraassay precision was estimated by using one batch of the test strips for replication analysis ($n = 3$) of the spiked OLA samples at 4, 12, and 36 μg/kg. For interassay precision, three batches of the test strips were used to detect the given samples. Precision was expressed as coefficient of variation (CV, %). OLA were added in before the samples were treated.

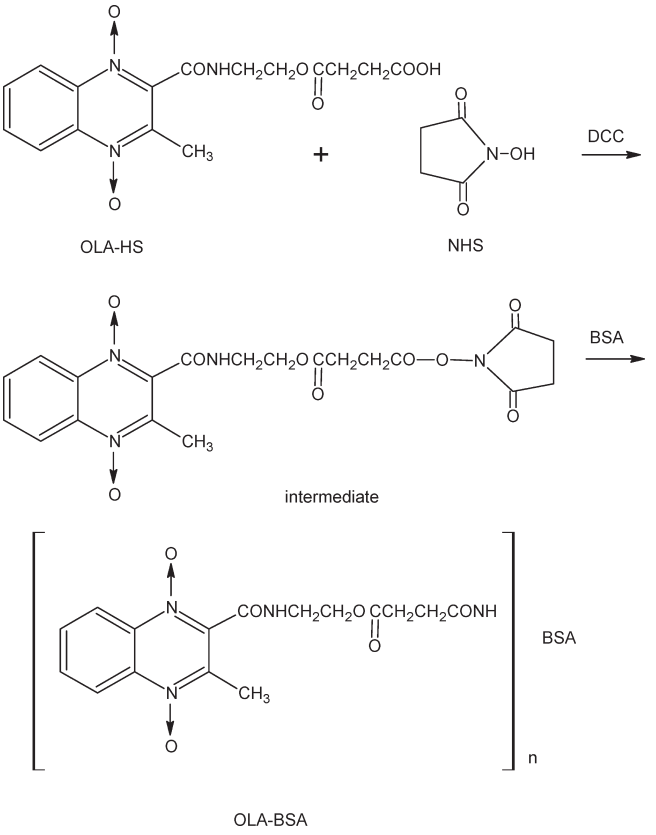

Figure 2. Chemical structures of the synthetic routes to OLA—BSA by the active ester method.

OLA Residue Detection and Comparison Study of HPLC and Test Strip Methods. OLA samples of pig urine and muscle tissue containing three different levels of analytes from market provided by the Supervision and Verification Center of the Ministry of Agriculture, Zhengzhou, China, were identified by GC—MS. The concentrations of the pig urine and muscle tissue samples were 2.90, 8.70, 21.30 ng/mL and 3.10, 8.60, 22.50 ng/mL for representation of the low, medium and high levels of the residues, respectively. The samples were analyzed in triplicate using a test strip and HPLC methods to compare the quantitative concordance of the two methods. A Student's *t* test statistical analysis was used to illustrate the difference between them.

RESULTS

Hapten Conjugation. OLA-HS, the derivative of olaquindox, was synthesized by the succinic anhydride method via its hydroxy and was then identified by MS (Figure 1). OLA-HS was coupled via its carboxyl group to the free amino groups of the protein carriers by the active ester method, resulting in the immunogen (OLA—BSA) and the coating antigen (OLA—OVA) (Figure 2). The conjugation ratio of OLA-HS to BSA and OVA was about 17.1:1and 12.4:1. The BALB/c mouse immunized with immunogen (OLA—BSA) showed higher titer (1:51200) and lower IC_{50} values (58.923 ng/mL); thus, it was chosen for subsequent experiments to cell fusion for the monoclonal antibody preparation.

Establishment of Hybridoma. Spleen cells from the mice immunized with OLA—BSA were fused with NS0 myeloma cells, and the resulting hybridomas were selected in HAT medium. One or more growing hybridomas were observed in almost all wells after 7 days. The supernatants from each well were screened

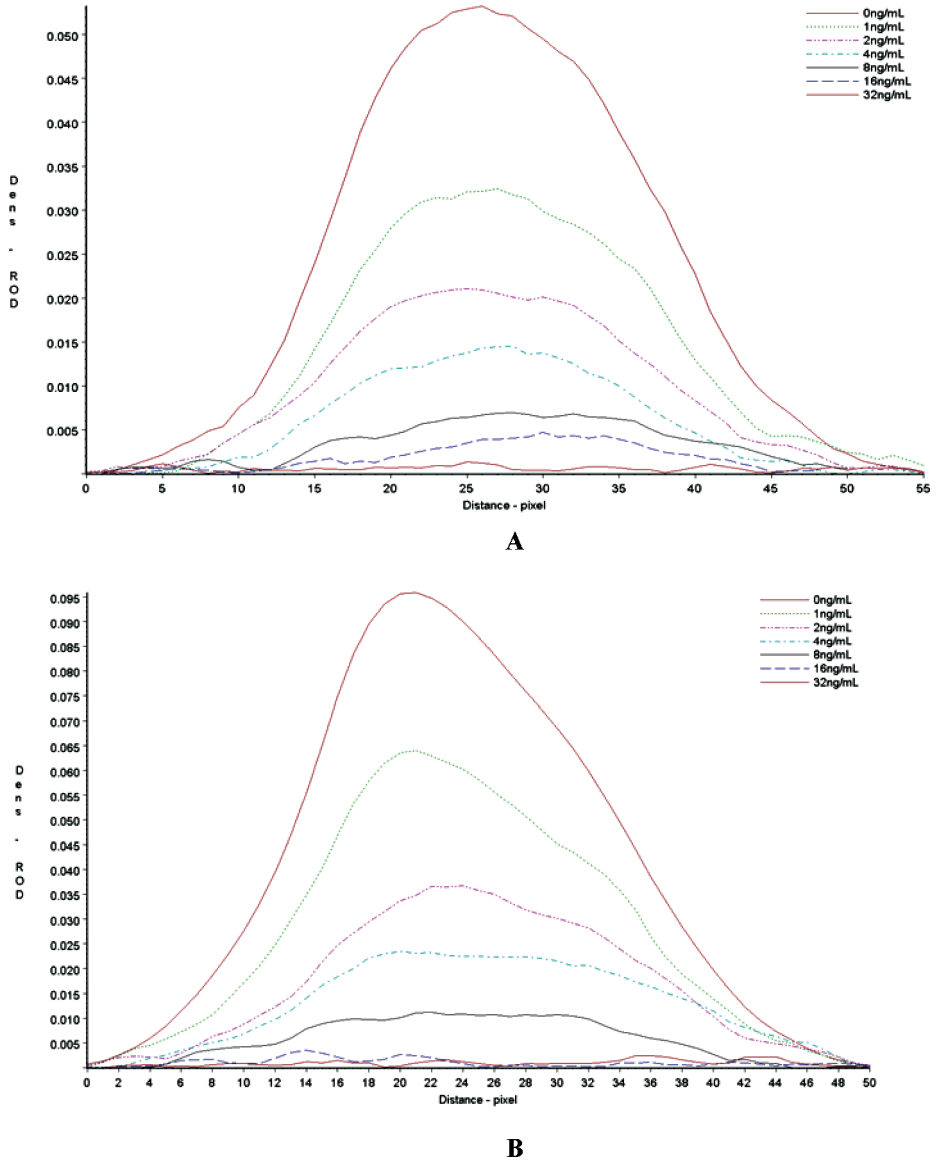

Figure 3. Relative optical density (ROD) curves of pig urine samples (A) and muscle tissue samples (B). The matrix extracts spiked with OLA standard solutions at 0, 1, 2, 4, 8, 16, and 32 ng/mL were tested using the test strips. Test lines were scanned with a TSR3000 membrane strip reader.

Table 1. G/Peak and G/D×Area of the Relative Optical Density (ROD) of Test Lines of the Pig Urine Samples[a]

OLA concn (ng/mL)	G/D×area-ROD (pixel)	G/peak-ROD (pixel)
0	101.1590	0.0544
1	60.8863	0.0334
2	46.2227	0.0214
4	32.6517	0.0157
8	20.1768	0.0075
16	9.9566	0.0051
32	1.0986	0.0020

[a] Standard OLA were added in the pig urine samples and tested using the test strips and the test lines scanned with a TSR3000 membrane strip reader.

for antibodies against OLA by a direct ELISA using microtiter plates coated with OLA−OVA conjugate. The cells from the wells showing the strongest response (OD > 2.0) were tested again in an indirect competitive ELISA for their ability to recognize free OLA. Five selected hybridomas were screened and further cloned by limiting dilution. After culture and further screening, the hybridomas 4E12 were intraperitoneally injected into mice to produce ascites fluid (mAb).

Characterization of Monoclonal Antibody. The titer of hybridoma cell line 4E12 was 1:5.12 × 10^5. The affinity constant (Ka) was 3.75 × 10^{10} L/mol, and the special mAb of 4E12 showed good sensitivity with IC_{50} of 1.51 ng/mL to OLA. The subclass of the mAb was identified as IgG1. Based on the ic-ELISA, the results of cross-reactivity demonstrate that the mAb has 4.72% cross-reactivity with carbadox and negligible cross-reactivity with other compounds (<0.01%) including maquindox, sulfadiazine, chloramphenicol, oxolinic acid, neomycin, tetracycline, penicillin, ractopamine, and sulfamonomethoxine.

Sensitivity of the Test Strip. The sensitivity of the test strip was determined by measuring the responses to OLA standard samples. Test lines were scanned with a Bio-Dot TSR3000

Table 2. G/Peak and G/D×Area of the Relative Optical Density (ROD) of Test Lines of the Pig Muscle Tissue Samples[a]

OLA concn	G/D×area-ROD (pixel)	G/peak-ROD (pixel)
0	102.2752	0.0959
1	63.3905	0.0641
2	47.5132	0.0367
4	33.5057	0.0235
8	21.2505	0.0112
16	9.9466	0.0035
32	1.0744	0.0016

[a] Standard OLA were added in the pig muscle tissue samples and tested using the test strips and the test lines scanned with a TSR3000 membrane strip reader.

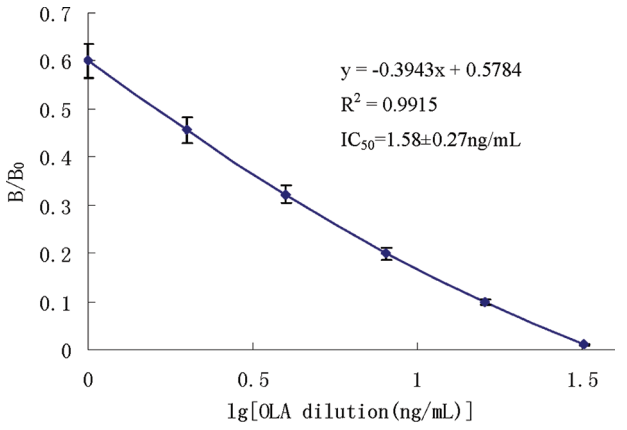

Figure 4. Standard curve in pig urine samples (A) and muscle tissue samples (B) for OLA using test strip detection. The *X*-axis is expressed as log concentration. B/B_0 represents the percentage of relative optical density (ROD) of standards divided by that of the ROD at 0 ng/mL. The linear regression correlation coefficient (R^2) is 0.9915 (A) and 0.9908 (B). IC_{50} was calculated as 1.58 ± 0.27 ng/mL (A) and 1.70 ± 0.26 ng/mL (B).

membrane strip reader. G/D×A (area) and G/peak of the relative optical density (ROD) decreased as the OLA concentration in the standard samples increased both in the pig urine samples (Figure 3A and Table 1) and muscle tissue samples (Figure 3B and Table 2).

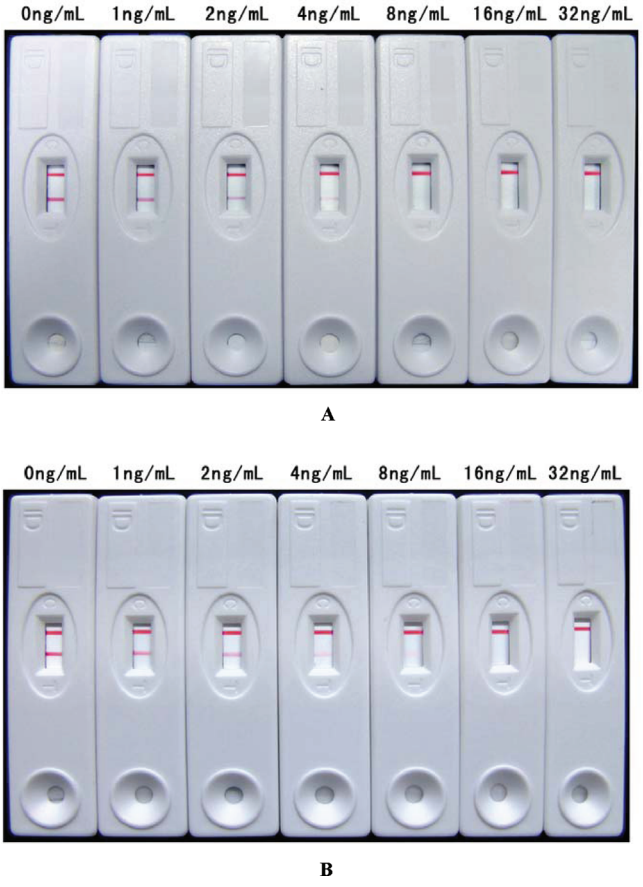

Figure 5. Standard OLA were added in pig urine samples (A) and muscle tissue samples (B) tested using the test strips and the test lines with the naked eye.

Table 3. Cross-Reactivity of Test Strip with Competitors

compound	IC_{50} (ng/mL)	cross-reactivity
olaquindox	1.58	100
carbadox	64	2.47
maquindox	$>2.0 \times 10^3$	<0.08
sulfamonomethoxine	$>2.0 \times 10^3$	<0.08
sulfadiazine	$>2.0 \times 10^3$	<0.08
oxolinic acid	$>2.0 \times 10^3$	<0.08
penicillin	$>2.0 \times 10^3$	<0.08
tetracycline	$>2.0 \times 10^3$	<0.08
chloramphenicol	$>2.0 \times 10^3$	<0.08
neomycin	$>2.0 \times 10^3$	<0.08
ractopamine	$>2.0 \times 10^3$	<0.08

The concentration of standard OLA and the G/D×area ROD produced a sigmoidal dose−response curve, which had good linearity within the range of 0.27−29.30 ng/mL and 0.31−29.06 ng/mL in pig urine samples and muscle tissue samples respectively. The regression equation was $y = -0.3943x + 0.5784$ (R^2 = 0.9915) (Figure 4A) and $y = -0.4051x + 0.5928$ (R^2 = 0.9908) (Figure 4B). LDL was quantitatively defined as the amount of OLA in the standard sample solution that caused a 20% decrease of the G/peak-ROD or G/D×area-ROD compared with that

Table 4. Recovery and Intra- and Interassay Precision of the Test Strips for OLA Spiked in Pig Urine and Muscle Tissue Samples[a]

	intraassay			interassay		
spiked OLA (μg/kg)	mean ± SD (μg/kg)	recovery (%)	CV (%)	mean ± SD (μg/kg)	recovery (%)	CV (%)
			Pig Urine Samples			
4	3.32 ± 0.17	83.0 ± 4.3	5.12	2.97 ± 0.22	85.8 ± 5.5	7.41
12	10.73 ± 0.34	89.4 ± 4.1	3.17	10.63 ± 0.54	88.6 ± 4.5	5.21
36	32.19 ± 1.04	89.4 ± 2.9	3.23	33.83 ± 1.37	94.0 ± 3.8	4.05
			Muscle Tissue Samples			
4	3.16 ± 0.19	79.0 ± 4.8	6.01	3.15 ± 0.24	78.8 ± 6.0	7.62
12	9.87 ± 0.46	82.3 ± 3.8	4.66	10.14 ± 0.61	84.5 ± 5.1	6.02
36	30.74 ± 2.35	85.4 ± 6.5	7.64	31.47 ± 2.28	87.4 ± 8.6	7.25

[a] Pig urine and muscle tissue samples were spiked with OLA at 4, 12, and 36 μg/kg. Intraassay precision was estimated by using one batch of the test strips ($n = 6$). For interassay precision, three batches of the test strips were used to detect the given samples. The recovery and coefficient of variation (CV, %) are calculated from triplicate assays in all cases.

Table 5. Comparison of a Test Strip with HPLC Methods for Three Levels of OLA Residues in Pig Urine and Muscle Tissue Samples

		test strip			difference between a test
level of OLA (ng/mL)	test strip (ng/mL)	difference (%)	HPLC (ng/mL)	HPLC difference (%)	strip and HPLC (%)
			Pig Urine Samples		
low, 2.90	2.77 ± 0.42	−4.48	2.81 ± 0.43	−3.10	−3.24
medium, 8.70	8.12 ± 2.35	−6.67	8.32 ± 3.49	−4.37	−5.68
high, 21.30	20.63 ± 3.16	−3.15	20.80 ± 3.07	−2.35	−1.07
			Muscle Tissue Samples		
low, 3.10	2.94 ± 0.56	−5.16	3.04 ± 0.38	−1.94	−4.12
medium, 8.60	8.07 ± 2.48	−6.16	8.26 ± 2.51	−3.95	−4.73
high, 22.50	22.17 ± 4.03	−1.47	22.82 ± 2.56	+1.42	−2.04

Three levels of pig urine and muscle tissue samples from market were identified by LC−MS and provided by the Supervision and Verification Center of Ministry of Agriculture, Zhengzhou, China. The samples were analyzed in triplicate using a test strip and HPLC methods. The differences of the test strip and HPLC compared with LC−MS were calculated, and the difference between the test strip and HPLC was also compared. Statistical analysis using a *t*-test did not show a significant difference between them.

pooduced by the blank sample. In qualitative testing with the naked eye, the LDL was determined by the minimal amount of OLA which produced a clearly visible difference in intensity of the test strip in comparison with the negative control line where no OLA was added in the sample. The result (about 2 ng/mL) was quantitatively close to the above (IC_{50}) 1.58 ± 0.27 ng/mL (Figure 5A) and 1.70 ± 0.26 ng/mL (Figure 5B).

Specificity of the Test Strips. When competitors at 2000 ng/mL (μg/kg) were tested, the color of the test line was the same as that of the negative control sample. The results of cross-reactivity demonstrate that this mAb has 2.47% cross-reactivity with carbadox and minimal cross-reactivity with other compounds (<0.08%) (Table 3). Therefore, the test strip for OLA was highly specific and showed negligible cross-reactivity to carbadox, maquindox, sulfadiazine, chloramphenicol, oxolinic acid, neomycin, tetracycline, penicillin, ractopamine, and sulfamonomethoxine.

Recovery of OLA in Pig Urine and Muscle Tissue Samples. To determine the accuracy, pig urine and muscle tissue extracts containing 4, 12, and 36 μg/kg of OLA were tested. The test was carried out in triplicate with a single batch of test strips and the optical density of the test line measured using the test strip reader and sample values calculated from the standard curve. As shown in Table 4, for intraassay reproducibility, recoveries were from 79.0 to 89.4% with the highest relative standard deviation at 7.6%. For interassay reproducibility, three different batches of the test strips were used for triplicate measurements of the samples. Recoveries were from 78.8 to 94.0% with the highest relative standard deviation at 7.6%.

Comparative Studies between Test Strips and LC−MS. A comparison between the test strip and the HPLC was performed with three levels of the actual samples. The differences of the test strip to actual samples were from 1.47 to 6.67%, and the HPLC was from 1.94 to 4.37%. The difference of the test strip and HPLC was from 1.07 to 5.68%. For both methods the results were almost identical, and statistical analysis using a *t* test did not show a significant difference between the two methods. The results from the two analysis methods showed good correspondence as shown in Table 5.

DISCUSSION

OLA, a small molecule with a molecular weight of 263.25, does not have immunogenicity. To prepare the immunogen and the

coating antigen, the treated molecule was conjugated to a protein carrier molecule. BSA and OVA were used as carriers. The moles of hapten coupled to the carrier protein are considered to be critical for the production of antibodies. Schneider believed the optimum ratio was 10−20:1,[33] Eilange considered the optimum ratio to be 5−25:1,[34] and Wust suggested the higher the ratio, the better.[35] The conjugation ratio of OLA-HS to BSA and OVA was about 17.1:1and 12.4:1. The titer and inhibition level of antiserum suggested that the immunogen has obtained a good immune response.

In this study, the IC_{50} of the test strip was 1.58 ng/mL and 1.70 ng/mL in different samples. However, the IC_{50} of the mAb OLA4E12 used to assemble the test strip was 1.51 ng/mL in a competitive ELISA. The reason for that may be the matrix effect when the extract leaks through the test strip. The specificity of the test strip was mainly determined by the nature of the mAb, so we only used pig urine samples for detection. For the quantitative assay, the optical density of a test line can be measured with a test strip reader, and according to the regression equation from the standard curve, the level of the OLA residue can be calculated. For semiquantitative and qualitative detection, the color of the test line can be evaluated directly by visual assessment. If the color of the test line was similar to that of the negative control sample, the sample was considered to be negative. If the color of the test line was within the range of the two definite control samples, the OLA concentration was considered to be between the two control samples. When the concentration of the OLA residue was greater and there was essentially no color observable at the test line, the sample should be interpreted as overproofed. By detecting different samples, the strips did not show significant differences.

The major advantages of the one-step strip test were that results could be obtained within 5−8 min and that all needed reagents were included in the strip. The strips could be used to detect the OLA residue in pig urine and muscle tissue in spots. This method provides a preliminary, semiquantitative result, which could be used to judge whether the OLA concentration remaining in the sample was higher than the detection limit or not.

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