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Monoclonal Antibody Production and the Development of an Indirect Competitive Enzyme-Linked Immunosorbent Assay for Screening Spiramycin in Milk

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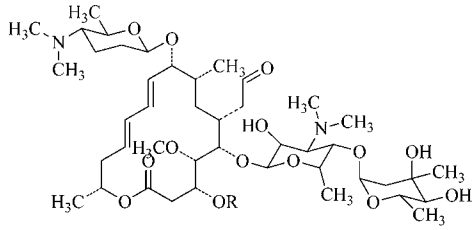
Supporting Information

ABSTRACT: To monitor spiramycin (SP) residue in milk, a monoclonal antibody (mAb)-based indirect competitive enzyme-linked immunosorbent assay (icELISA) was developed. This study described the preparation of three immunogens and the production of a high-affinity mAb. After optimization, the 50% inhibition concentration (IC_{50}) for the developed icELISA was estimated as 0.97 ng/mL in the assay buffer, and the limit of detection and limit of quantitation were 2.51 and 4.40 μg/L in the milk matrix. The newly developed assay demonstrated negligible cross-reactivity with 15 other macrolide antibiotics, but not with kitasamycin (23.4%). The mean recoveries ranged from 81 to 103% for the spiked samples (5, 10, and 50 μg/L), and the coefficient of variation ranged from 5.4 to 9.6%. The icELISA was validated by LC-MS/MS method, and all results demonstrated that it was a suitable screening method for detecting SP residue in milk without requiring a cleanup process.

KEYWORDS: *spiramycin, monoclonal antibody, immunoassay, residue, milk*

INTRODUCTION

Spiramycin (SP, Figure 1) is a multicomponent macrolide antibiotic that is widely used as a veterinary drug and feed additive[1] and largely consists of three closely related substances: SPI is the major component and contributes approximately 63% of the mixture, while SPII and SPIII contribute approximately 24 and 13%, respectively.[2] Spiramycin is highly active against gram-positive bacteria and mycoplasma species.[3] Therefore, SP is widely used to treat toxoplasmosis and other soft-tissue infections in cattle, swine, poultry, and sheep.[4]



R=H Spiramycin I $C_{43}H_{74}N_2O_{14}$
R=CO-CH_3 Spiramycin II $C_{45}H_{76}N_2O_{15}$
R=CO-CH_2-CH_3 Spiramycin III $C_{46}H_{78}N_2O_{15}$

Figure 1. Chemical structure of spiramycin.

The incorrect use of SP and the disregard for withdrawal time after treatment result in SP residues in food, which may have allergic and toxic effects on consumer health.[5] Furthermore, SP residues in foods of animal origin are related to the development of antibiotic-resistant bacterial strains in humans.[6] To protect consumer health, the SP residues in foods of animal origin must be monitored. The European Union, United States, and China have already set maximum residue levels (MRLs) for SP that range from 100 to 1000 μg/kg in various food matrixes.[2,3,7]

During the last two decades, high-performance liquid chromatography[8,9] and liquid chromatography-tandem mass spectrometry[10−12] (LC-MS/MS) have been widely used to measure the macrolide antibiotic residues in various foods of animal origin.[13,14] These methods are typically labor-intensive, time-consuming, and require sophisticated instruments; thus, they are unsuitable for screening large numbers of samples.[10] However, immunoassays, especially enzyme-linked immunosorbent assays (ELISA), have been confirmed as alternative screening methods for rapid analysis[15,16] without some of the limitations of chromatographic methods.[17,18] The ELISA method could simply and reliably provide high sample throughput, sensitivity, and selectivity.[19,20] To the best of our knowledge, no other reports have yet been published concerning the development of an immunoassay to detect SP residues in food. In this paper, we detail the production of highly specific mAbs and the development of an icELISA to screen for SP residues in milk without requiring complicated sample preparation.

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MATERIAL AND METHODS

Reagents and Apparatus. Erythromycin A (91.6%), spiramycin (94%), josamycin (98%), and clarithromycin were purchased from TCI Chemicals (Shanghai, China). The azithromycin (100%), tulathromycin (100%), and desosaminylazithromycin (100%) reference standards were purchased from Pfizer Pharmaceuticals Limited (Brooklyn, NY, USA). Valnemulin and tiamulin were purchased from the Council of Europe's European Pharmacopoeia. Tylosin and tilmicosin (93.7%) were purchased from the China Institute of Veterinary Drug Control (Beijing, China). Kitasamycin and acetylspiramycin (90.2%) were purchased from the China Pharmaceutical Biological Products Analysis Institute (Beijing, China). Erythromycylamine was purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Avermectin, roxithromycin (97%), and erythromycin ethyl succinate (98%) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Dirithromycin (>95%) and oleandomycin triacetate were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Bovine serum albumin (BSA), ovalbumin (OVA), *o*-carboxymethoxylamine hemihydrochloride (CMO), succinic anhydride, carbonyldiimidazole, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, *N*-hydroxy succinimide, and Freund's adjuvant were also purchased from Sigma-Aldrich (St. Louis, MO, USA). Horseradish peroxidase-conjugated goat anti-mouse IgG was provided by Jackson ImmunoResearch (West Grove, PA, USA). A SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA) was used for this study.

Synthesis of the Immunogen and Coating Antigen. *o-Carboxymethoxylamine Method:* Spiramycin (SP, 8.5 mg) was dissolved in 6 mL of absolute ethanol, and *o*-carboxymethoxylamine (CMO, 22.0 mg) dissolved in 2 mL of pure water was added dropwise. The pH of the reaction mixture was adjusted to 5.5 using 1 M $NaHCO_3$, and the mixture was maintained at 50 °C for 5 h. Twenty milliliters of dichloromethane was added to extract the hapten, and the organic phase was vacuum-dried. The as-obtained hapten SP-CMO was dissolved in dimethylformamide and confirmed via mass spectrometry. Then, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (3.8 mg) and *N*-hydroxy succinimide (2.3 mg) were added, and the mixture was stirred for 4 h at room temperature. The activated SP-CMO solution was added dropwise to a BSA or OVA solution under magnetic stirring for 12 h at room temperature. The resulting immunogen and coating antigen, named SP-CMO-BSA or SP-CMO-OVA, respectively, were dialyzed against 0.01 M PBS for 3 days at room temperature then stored at −20 °C until use.[12]

Carbonyldiimidazole Method: The spiramycin (SP, 8.0 mg) and carbonyldiimidazole (CDI, 4.0 mg) were dissolved in 2 mL of acetone. The mixture was stirred at room temperature for 3 h to form an activated amide intermediate. Then, the reaction mixture was vacuum-dried, and the yellow powder was dissolved in 1 mL of dimethylformamide. This solution was added dropwise to 10 mg of carrier protein dissolved in 5 mL of carbonate buffer (0.05 M, pH 9.5). After stirring for 5 h at room temperature, the conjugates—SP-CDI-BSA and SP-CDI-OVA—were dialyzed against 0.01 M PBS for 3 days at room temperature, then stored at −20 °C until use.[21]

Succinic Anhydride Method: SP (8.5 mg) and succinic anhydride (HS, 4.0 mg) were dissolved in 2 mL of pyridine and gently stirred for 2 h at room temperature. After evaporating the pyridine, the residue was dissolved in 2 mL of *N*,*N*-dimethylformamide. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (3.8 mg) and *N*-hydroxy succinimide (2.3 mg) were added. The mixture was gently stirred for 4 h at room temperature and then added dropwise to a carrier protein solution. After being stirred for 5 h at room temperature, the conjugates—SP-HS-BSA and SP-HS-OVA—were dialyzed against 0.01 M PBS for 3 days at room temperature and stored at −20 °C until use.

The coupling ratios (the number of hapten molecules per molecule of carrier protein) of the conjugates were identified via MALDI-TOF/MS (Bruker, Daltonics, Billerica, MA) by College of Biological Science (China Agricultural University, Beijing, China) and calculated according to the following equation:[22]

$$\text{coupling ratio} = (MW_{conjugate} - MW_{protein})/MW_{hapten}$$

Monoclonal Antibody Production. Eight female BALB/c mice were immunized with each of the three immunogens (at a dosage of 50 μg protein per mouse) for mAb production.[23,24] The mice were immunized subcutaneously using an emulsion of the immunogen in Freund's complete adjuvant. After 2 weeks, the mice were boosted every 2 weeks. Then, serum was collected from each mouse, and the antibody titer was monitored. The mouse with the highest antibody titer was sacrificed, and the splenocytes were fused using SP2/0 myeloma cells. The fused cells were propagated in HAT medium. After fusion, the cells were selected against unfused cells using HAT medium. Following the selection of the fused cells, the HAT medium was replaced by HT medium. The growing hybridoma cells were screened for antibody production using a noncompetitive indirect ELISA. The hybridomas that produce a specific mAb against SP were subcloned twice via the limiting dilution method. The subcloned hybridoma cells were collected, centrifuged, and frozen in liquid nitrogen. The ascites from the hybridoma-induced mice were purified using saturated ammonium sulfate precipitation and used to develop the icELISA.

Indirect Competitive ELISA. The icELISA approach can be described as follows:[25,26] each well of a microtiter plate was coated with 100 μL of coating antigen. The plates were incubated overnight at 4 °C, then blocked with 150 μL of blocking buffer (0.01 M PBS containing 0.5% casein). After washing, 50 μL of the optimal antibody dilution and 50 μL of the SP standard with serial dilutions (0.03−81 ng/mL) were added to the wells, and the plates were incubated for 30 min at 37 °C. After washing as described, 100 μL of diluted goat anti-mouse IgG-HRP solution was added, and the plates were incubated for 30 min at 37 °C. After further washing, 100 μL of TMB substrate (0.1% 3,3′,5,5′-tetramethylbenzidine and H_2O_2 in 0.05 M citrate buffer, pH 4.5) was added, and after incubation at 37 °C for 15 min, the reaction was terminated using 2 M H_2SO_4. Then, the absorbance values were measured on an ELISA plate reader at 450 nm.

The comparative experiments were carried out via checkerboard titration to determine the suitable immune reagent concentrations. The experimental conditions (organic solvent tolerance, ionic strength, and pH of the assay buffer) were optimized according to our previous studies.[25,27]

Organic Solvent Effect: Calibration curves were obtained using the SP standards and the mAb dissolved in PBS buffer (containing various proportions of methanol and acetonitrile). The effect of the organic solvent on the icELISA performance was assessed.

Ionic Strength Effect: The SP standards and the mAb were dissolved in PBS buffers of varying ionic strength, which were prepared by adding NaCl (from 0 to 0.5 M) to the PBS buffer solution. The effect of the assay buffer ionic strength on the performance of the icELISA was assessed.

pH Effect: The SP standards and the mAb were dissolved in PBS buffers of differing pH values (from 5.5 to 9.5). The effect of the assay buffer pH on the icELISA performance was assessed.

Determining the Cross-Reactivity. The absorbance of the wells that contained only assay buffer was referred to as B_0. The absorbance of the standards was normalized against the absorbance of the assay buffer (B/B_0). The standard concentration at the midpoint of the standard curve (IC_{50}) was defined as the concentration of the competitor that inhibited the binding of the antibody by 50% (B/B_0 = 50%). The competition curves were fitted to a four-parameter logistic equation,[28] from which the IC_{50} values were calculated.

$$Y = (A - D)/[1 + (X/C)^B] + D$$

in which A = response at high asymptote, B = slope factor, C = the concentration corresponding to 50% specific binding (IC_{50}), D = the response at low asymptote, and X = calibration concentration.

To determine the icELISA specificity, a cross-reactivity study was carried out. The cross-reactivity of the antibodies against other macrolide antibiotics was calculated using the following equation:

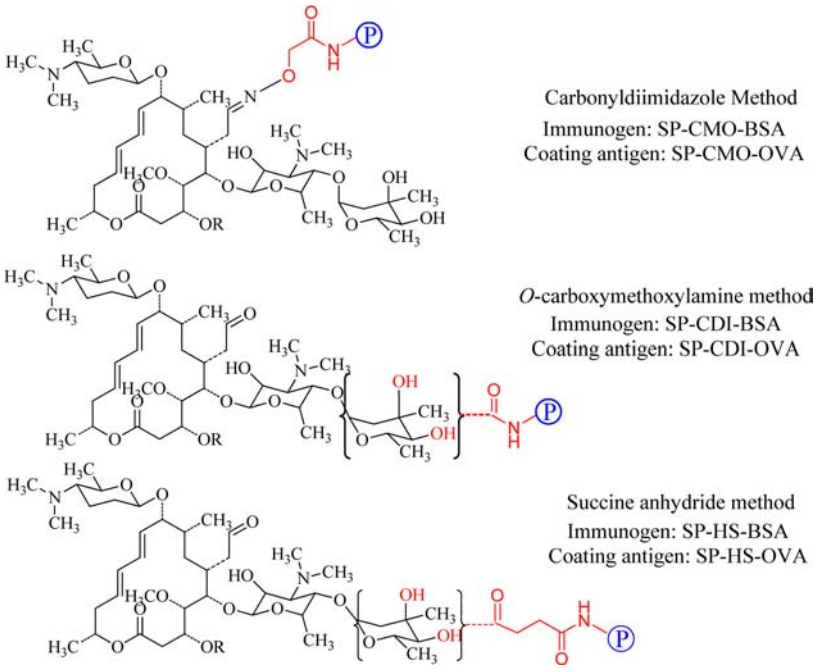

Figure 2. Chemical structures of the synthesized immunogens and coating antigens.

Table 1. Coupling Ratios of the Hapten–Protein Conjugates

conjugate	SP-CMO-BSA	SP-CDI-BSA	SP-HS-BSA	SP-CMO-OVA	SP-CDI-OVA	SP-HS-OVA
coupling ratio	4.5	3.2	3.4	6.8	4.2	5.7

$$\text{cross-reactivity}\ (\%) = (IC_{50}\ \text{of SP})/(IC_{50}\ \text{of other analytes}) \times 100$$

Sample Preparation. Blank milk samples were provided by the Veterinary Drug Safety Inspection & Testing Center of the Ministry of Agriculture (Beijing, China) and stored in a refrigerator (4 °C) prior to use. Each sample was tested via LC-MS/MS prior to the spiking and recovery tests. One milliliter samples of milk were diluted 10-fold with PBS solution, and 50 μL of each diluted solution was used in the icELISA.

Assay Validation. The icELISA method was validated as an assay according to the relevant sections of the Commission Decision 2002/657/EC[29] and CODEX Guidelines.[30] The limit of detection (LOD) was based on the mean value of 20 blank samples plus three times the mean standard deviation. The limit of quantification (LOQ) was estimated as the mean of the same results plus 10 times the standard deviation. The accuracy and precision of the method were represented by the recovery and coefficient of variation (CV), respectively. The mean recovery and CV values were calculated via the repeated analysis of the spiked samples (5, 10, and 50 μg/kg). The intra-assay CV was measured using four replicates of each spiked concentration, while the inter-assay CV was based on the results from 4 different days. Finally, the developed ELISA was validated by the LC-MS/MS method[31] using spiked milk samples.

RESULTS AND DISCUSSION

Synthesis of Immunogen and Coating Antigen. The hapten design and the synthesis of the required immunogens are critical to the success of rapid immunoassays. Because it is a small molecule, SP lacks immunogenicity. To generate a specific antibody, SP should be conjugated with macromolecules to elicit an immune response via an active group, such as a carboxyl or amino group. As shown in Figure 1, two hydroxyls and one ketone group are present in the structure of SP and can theoretically be derivatized to link with carrier proteins (BSA and OVA in this study).

In this study, three new SP haptens were synthesized via the derivatization of SP with CMO, CDI, and HS individually. Therefore, three different types of spacer arms were introduced onto the SP hapten. The carbonyldiimidazole method introduced a one-carbon spacer arm, and the *o*-carboxymethoxylamine method introduced a short spacer arm containing two carbons. The succinic anhydride method introduced a long spacer arm containing four carbons on the hapten. The chemical structures of the synthesized immunogens and coating antigens containing three different spacer arms are provided in Figure 2. The successful synthesis of the three immunogens and coating antigens was confirmed using MALDI-TOF/MS analysis.[22] The coupling ratios of the conjugates are provided in Table 1.

Production of the mAbs. SP-CMO-BSA, SP-CDI-BSA, and SP-HS-BSA were used to immunize the mice for mAb production, while SP-CMO-OVA, SP-CDI-OVA, and SP-HA-OVA were used as coating antigens to develop the icELISA. Ten days after the fourth injection, the antiserums were isolated and measured via icELISA. The antiserum sensitivities were characterized by calculating the IC_{50} values of the antisera from each mouse after evaluating the antibody titer. Of the immunized mice, the highest antibody titer and sensitivity were achieved with the immunogens SP-CMO-BSA and SP-HS-BSA. No antibody titer was determined for the antiserum that used SP-CDI-BSA as the immunogen. Compared with the three synthesized haptens, the spacer arms CMO and HS appeared preferable for maintaining a fully exposed hapten on the surface of the carrier protein. This approach proved beneficial by exposing a specific region of the hapten to the animals' immune systems.[32]

Table 2. Curve Parameters Obtained Using Mouse Antisera Collected after the Fourth Injection[a]

		mouse no.							
immunogen	parameters	1	2	3	4	5	6	7	8
SP-CMO-BSA	antiserum dilution	6000	3000	3000	1500	1500	1500	1500	1500
	antigen dilution	3000	3000	3000	3000	300	3000	3000	3000
	maximum absorbance (B_0)	1.387	1.612	1.407	1.687	1.883	0.787	1.365	0.725
	IC_{50} (ng/mL)	2.5	18.4	2.4	5.3	47.8	9.2	21.9	13.8
SP-HS-BSA	antiserum dilution	800	800	1000	1000	1000	1000	400	1000
	antigen dilution	1000	1000	1000	1000	1000	1000	1000	1000
	maximum absorbance (B_0)	1.415	2.000	2.121	1.007	0.991	1.672	1.221	2.199
	IC_{50} (ng/mL)	8.5	7.6	>1000	>1000	>1000	>1000	4.6	>1000
SP-CDI-BSA	no antibody titer was determined for the eight mice								

[a]Mean values of three independent determinations.

Table 3. Assay Parameters of the Five mAbs Obtained from Mice Immunized with SP-CMO-BSA Using Spiramycin as the Competitor in the icELISA[a]

antibody	3B10	5E11	1G12	2F1	6E2
antibody dilution	1/50000	1/50000	1/400000	1/100000	1/200000
coating antigen dilution	1/60000	1/100000	1/200000	1/100000	1/200000
OD_{max}	1.772	1.543	1.742	1.841	1.552
IC_{50} (ng mL^{-1})	1.2	1.5	2.0	5.1	2.2

[a]Mean values of three independent determinations.

The antisera parameters obtained from each mouse at the last bleeding are provided in Table 2. Generally, the mice that received SP-CMO-BSA as the immunogen had a higher antibody titer and lower IC_{50} values than those immunized with SP-HS-BSA. All eight mice immunized with SP-CMO-BSA exhibited an antisera dilution above 1/1500, whereas all mice immunized with SP-HS-BSA exhibited an antisera dilution below 1/800 with suitable absorbance values (Table 2). The results summarized in Table 2 indicate that all mice receiving SP-CMO-BSA presented an affinity to SP, with IC_{50} values ranging from 2.4 to 47.8 ng/mL; however, in mice that received SP-HS-BSA, only three out of eight had an affinity to SP, with IC_{50} values ranging from 4.6 to 8.5 ng/mL.

Mouse number 3 in the SP-CMO-BSA group and mouse number 7 in the SP-HS-BSA group were used to produce monoclonal antibodies due to their relatively higher sensitivity. Five hybridomas producing specific mAbs against SP were obtained using SP-CMO-BSA as the immunogen, and the mAbs were named 3B10, 5E11, 1G12, 2F1, and 6E2. No hybridomas were successfully produced using SP-HS-BSA as the immunogen. The mAbs were prepared and characterized, and the results are provided in Table 3. The optimum dilution of 3B10 was 1/50000, which is 16.7 times higher than that with the corresponding antiserum, while the sensitivity (IC_{50}) of the mAb was increased 2-fold over that of the antiserum.

icELISA Method. The as-synthesized coating antigens and the three antibodies were screened and characterized using homologous and heterologous formats to select the best combinations. Three coating antigens (SP-CMO-OVA, SP-CDI-OVA, and SP-HS-OVA) were evaluated in the optimization of the icELISA method. As observed in Figure 3, the heterologous coating antigen SP-CDI-OVA significantly improved the sensitivity of the immunoassay by reducing its nonspecific recognition (toward cross-linkers). Therefore, this combination (SP-CDI-OVA/mAb 3B10) was used for all subsequent experiments.

A checkerboard titration was performed to determine the optimal dilution of the coating antigen SP-CDI-OVA and mAb

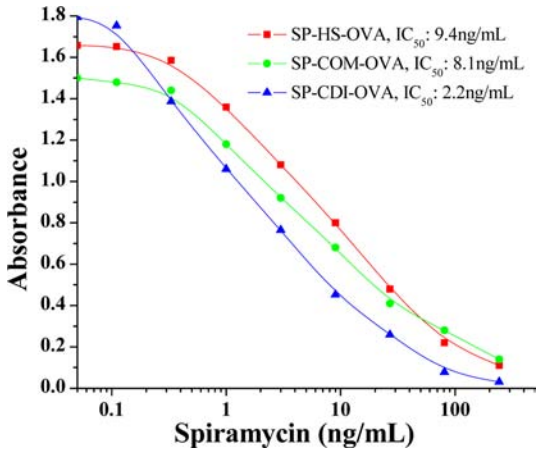

Figure 3. Comparison of the three coating antigens used in the icELISA optimization with mAb 3B10.

3B10. To establish a sensitive icELISA method, a low IC_{50} value and an absorbance value from 1.5 to 2.0 were deemed suitable for the following experiments. The optimal dilutions of the mAb 3B10 and the coating antigen SP-CDI-OVA were 1:60000 and 1:50000, respectively.

The performance of immunoassays is partially dependent on the microenvironment surrounding the antibody−antigen interaction. Some physical and chemical conditions can influence the antigen−antibody interaction, such as the pH value, ion strength, and organic solvents.

Organic Solvent Effect: According to previous reports, the organic solvents in the assay buffer significantly influence the parameters of the immunoassay (such as the maximum absorbance, IC_{50}). Optimizing these conditions can improve the sensitivity of the assay by reducing any nonspecific interactions, that is, remove the background noise. The veterinary drugs were dissolved in organic solvents to yield a stock solution, and the working solutions were prepared via serial dilutions with a buffer prior to the immunoassay. Finally,

Table 4. Optimized Parameters for the ELISA

parameter		B_0	IC_{50} (ng/mL)	IC_{50}/B_0	parameter		B_0	IC_{50} (ng/mL)	IC_{50}/B_0
methanol tolerance	**0**	**1.870**	**3.35**	**1.791**	acetonitrile tolerance	**0**	**2.326**	**2.39**	**1.028**
	1%	1.688	4.22	2.500		1%	2.271	2.92	1.286
	5%	1.406	6.08	4.324		5%	1.595	5.16	3.235
	10%	0.660	6.10	10.17		10%	0.900	15.46	17.18
	20%	0.455	14.59	32.07		20%	0.240	25.10	104.5
	50%	0.234	*a*	*a*		50%	0.170	*a*	*a*
ionic strength	0	1.750	16.04	9.166	pH	5.5	2.058	4.40	2.138
	0.05	1.646	9.87	6.384		**6.5**	**2.013**	**3.75**	**1.863**
	0.15	1.347	5.31	3.942		7.0	1.953	5.22	2.673
	0.2	1.308	4.53	3.463		7.5	1.555	5.39	3.466
	0.3	**1.256**	**2.56**	**2.038**		8.5	1.108	5.03	4.540
	0.5	1.245	2.88	2.313		9.5	1.086	9.19	8.462

[a] IC_{50} values cannot be calculated in this situation.

the organic solvent was optimized to inform the sample treatment because the target analyte in the samples is typically extracted using an organic solvent. Therefore, an investigation was conducted to determine the effect of the quantity of organic solvent in the buffer on the immunoassays. The effects of the organic solvents (methanol and acetonitrile) on the icELISA performance were also examined. As indicated in Table 4, the maximum signal (B_0) and sensitivity clearly decreased when the concentrations of methanol and acetonitrile in the assay buffer increased. When the concentrations of methanol and acetonitrile exceeded 10%, the absorbance values were poorly fitted to a sigmoidal curve. Even minimal solvent quantities (1%) had a negative effect on the icELISA performance; thus, the assay buffer without an organic solvent was used in the icELISA.

Ionic Strength Effect: To evaluate the effect of ionic strength, the immunoassay performance was investigated in PBS with an NaCl concentration ranging from 0 to 0.5 M. As observed in Table 4, the B_0 value was 1.750 in the absence of salts and decreased slightly as the NaCl concentration increased. The NaCl concentration also affected the sensitivity of the assay, whose IC_{50} values ranged from 2.56 to 16.04 ng/mL. A NaCl concentration of 0.3 M was used in the assay buffer, demonstrating a compromise between the B_0 value and the sensitivity (IC_{50}).

pH Effect: To assess the influence of pH on the assay performance, competitive curves for SP were obtained in PBS at pH values ranging from 5.5 to 9.5. The results in Table 4 indicate that the B_0 value of the ELISA decreased slightly when the pH increased from 5.5 to 9.5. These results indicate that the immunoassay became less stable when the pH of the buffer changed from acidic to alkaline. Considering the B_0 value and sensitivity (IC_{50}), a slightly acidic pH (pH 6.5) is preferred for the icELISA buffer.

After these optimizations, competitive inhibition standard curves for SP were prepared accordingly. The calibration curves are in the range of 0.01−81 ng/mL (see Figure 1 in the Supporting Information), and the IC_{50} of the icELISA was 0.97 ng/mL after optimization.

Cross-Reactivity. The specificity of the icELISA was evaluated by determining the cross-reactivity using a set of structurally related macrolide antibiotics. The IC_{50} and cross-reactivity values are summarized in Table 5. The icELISA demonstrated low cross-reactivity with kitasamycin (23.4%), acetylspiramycin (1.2%), and josamycin (1.2%) and negligible cross-reactivity (<0.1%) with the other macrolide antibiotics tested.

Table 5. IC_{50} and Cross-Reactivity Values of the mAb 3B10 Using SP-CDI-OVA as the Coating Antigen

analyte	IC_{50} (ng/mL)	cross-reactivity (%)
spiramycin	0.97	100
acetylspiramycin	82.90	1.2
kitasamycin tartrate	4.15	23.4
josamycin	79.51	1.2
other macrolide antibiotics[a]	>1000	<0.1

[a] Other macrolide antibiotics include erythromycin, erythromycin ethylsuccinate, erythromycylamine, dirithromycin, roxithromycin, clarithromycin, oleandomycin, azithromycin, desosaminylazithromycin, tularaemia, eprinomectin, tylosin, tilmicosin, valnemulin, tiamulin, avermectins, ivermectin, doramectin, and emamection benzoate.

Sample Preparation. In comparison with the confirmation methods, the primary advantage of ELISA is that it does not require complicated sample preconcentration and cleanup procedures. However, the matrix effect is a common challenge of immunoassays for food analysis that can reduce the sensitivity and reliability of the immunoassay by reducing the color development or interfering with the antigen−antibody binding. To evaluate the matrix effects, the standard curves generated in PBS were compared with those obtained using the diluted matrix. As shown in Figure 4, the matrix effects could be

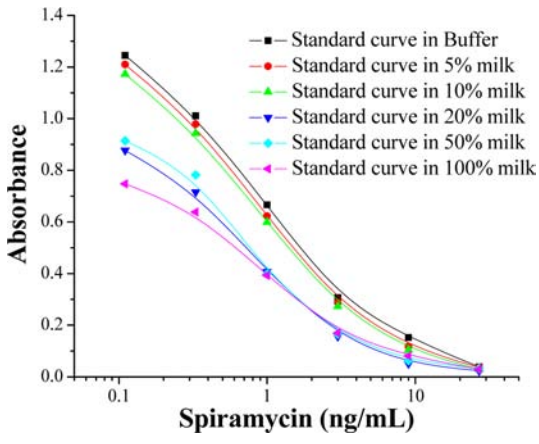

Figure 4. Evaluation of the matrix effect in the milk samples.

reduced to insignificant levels when the control milk sample was diluted 10 or 20 times. Although a special cleanup procedure (such as organic solvent extraction) may achieve better results, the dilution method is simple and suitable for on-site detection. Therefore, the milk samples were diluted 10-fold prior to screening.

Assay Validation. The assay was validated by investigating the LOD, LOQ, accuracy, repeatability, and reproducibility. By evaluating 20 different blank samples, the LOD and LOQ of the as-developed icELISA was 2.51 and 4.40 μg/L, which is far below the MRLs set by the European Union, United States, and China. Other researchers have reported[8−14] that their instrumental methods offered LOD values ranging from 1 to 24.1 μg/kg. The LOD and LOQ of the new icELISA were comparable to that of the previously reported HPLC or LC-MS/MS methods. To evaluate the accuracy and precision of the proposed ELISA, a spiked recovery analysis was conducted. When the blank samples were spiked at 5, 10, and 50 μg/L, the mean recovery ranged from 81 to 103%, with intra-assay and inter-assay CVs ranging from 5.4 to 9.6%. The results of the accuracy and precision tests are provided in Table 6. These data

Table 6. Mean Recovery and Coefficient of Variation (CV) for Detecting SP Residues in Milk (n = 4)

	intra-assay		inter-assay	
spiked (ng/mL)	mean recovery (%)	CV (%)	mean recovery (%)	CV (%)
5	98 ± 5.3	5.4	85 ± 6.2	7.3
10	81 ± 5.5	6.8	92 ± 6.3	6.8
50	87 ± 7.2	8.3	103 ± 9.9	9.6

meet the requirements of an analytical method for residue analysis, and the icELISA method satisfied both the repeatability and reproducibility requirements. In 2006, García-Mayor et al.[8] reported an HPLC method with an LOD of 24.1 μg/kg for spiramycin in sheep milk and CVs ranging from 1 to 6.5%. In 2008, Maher et al.[9] described an HPLC method with an LOD of 5 μg/kg and CVs ranging from 1.2 to 6.8%. In 2008, Berrada et al.[10] reported an LC-MS/MS method with a quantification limit of 25 μg/kg for animal muscle and CVs below 12%. The detection limits of the LC-MS/MS methods[11−14] were in the range of 1−20 μg/kg, with CV values below 20%. Thus, we think the ELISA we developed is at least comparable to the other published methods (HPLC, LC-MS/MS) in its sensitivity and precision.

Contaminated milk samples were prepared at the Veterinary Drug Safety Inspection and Testing Center of the Ministry of Agriculture (Beijing, China) by spiking fresh milk samples with the appropriate standards, and the contaminated samples were used for the final validation of the ELISA method. The results of the icELISA were compared to LC-MS/MS analysis using a correlation test. As can be observed from Figure 5, results measured by the ELISA were compatible with that of the LC-MS/MS method, and the coefficient of correlation R^2 was 0.96. These results demonstrate that the as-developed ELISA has the potential for use in milk sample analysis prior to confirmation by a reference method.

Immunoassays are commonly used for the rapid screening of veterinary drug residues, but the use of an immunoassay to determine SP residues is rare. In this study, new SP immunogens were prepared and used to produce sensitive and specific mAbs. Based on the as-produced mAb, a

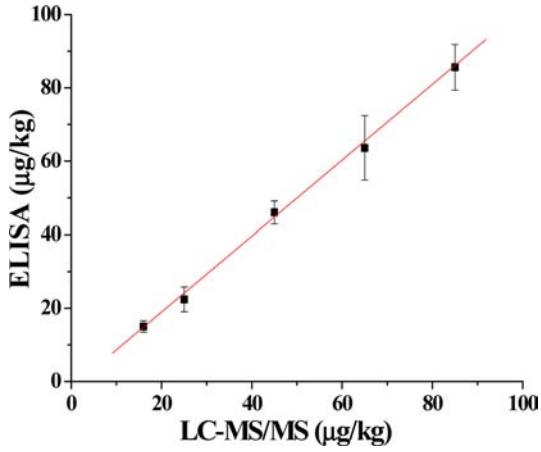

Figure 5. Correlation of the ELISA and LC-MS/MS methods. The ELISA data represent the mean values of triplicate measurements from a single experiment; error bars represent the standard error of triplicate measurements.

heterologous icELISA was developed to detect SP residues in milk that could provide a practical tool for the routine screening of large numbers of milk samples, with positive results confirmed via reference methods.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental Figure 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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