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ARTICLE

Cephalexin Residue Detection in Milk and Beef by ELISA and Colloidal Gold Based One-Step Strip Assay

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An evaluation of a rapid enzyme-linked immunosorbent assay (ELISA) and colloidal gold based one-step strip assay for cephalexin (CEX) residue detection in milk and beef is described. A monoclonal antibody (mAb) against CEX was produced using cephalexin−bovine serum albumin (CEX-BSA) conjugate as the immunogen, which exhibited no cross-reactivity with applied chemicals in the studied concentration range. The detection limit of rapid ELISA was calculated as 0.39 μg/kg in PBS and 19.5 μg/kg in beef and milk, which was quite lower than the European Union Maximum Residue Limit (MRL) of 100 μg/kg in milk and 200 μg/kg in muscle. Spiked samples were detected with a mean recovery of 82.8−124% and coefficient of variation of 4.88−25%, which indicated a good agreement with the spiked concentration. Accuracy and reproducibility were determined using spiked samples with four different final concentrations of 1, 2, 5, and 10 μg/kg of CEX (n = 7). Mean intra-assay variation of 6.67% and inter-assay variation of 10.66% were obtained. In contrast, the strip test for CEX had a visual detection limit of 0.5 μg/kg, which could be evaluated within 3−10 min. However, positive samples should be further quantified by more sensitive and accurate competitive indirect ELISA method. In conclusion, the described strip test is rapid, simple, and cost-effective as well as sensitive and specific enough for reliable and accurate on-site screening.

KEYWORDS: Cephalexin; monoclonal antibody; colloidal gold; enzyme-linked immunosorbent assay; milk and beef

INTRODUCTION

Occurrence of antimicrobial drug residues in food carries potential risk by selection of resistant pathogenic organisms and causes adverse effects on intestinal microflora and decreases the quality of animal products. Direct or indirect impacts on human health made it imperative to develop analytical methods. Accumulation of residues are mainly in products of animal origin such as milk and beef (*1*−*4*).

Typical primary screening tests based on microbial inhibition and bacterial receptor assays are qualitative or semiquantitative. Mostly, they are not specific due to various effects from dietary sources, animal diseases, or other variables (*5*). In recent years, there is a trend to develop quick, simple, and high throughput immunochemical and enzymatic assays based on antigen−antibody interaction (*1*, *2*, *5*). Disadvantages are cross-reactivities due to less specificity of the antibodies (*6*−*8*). Quantitative methods commonly used are chromatographic techniques such as high performance liquid chromatography (HPLC)−tandem mass spectrometry (MS). Nevertheless, sensitivity and specificity are high, but expertise and complicated sample preparation are required (*2*).

Cephalexin (CEX) is a potent antibiotic, belonging to β-lactams, commonly used in veterinary medicine in some countries. Residues of CEX in food products are considered a high risk to the consumer (*9*). In the European Union (EU), maximum residue limit (MRL) for CEX in milk, muscle, fat, liver, and kidney are 100, 200, 200, 200, and 1000 μg/kg, respectively (*10*).

Detection methods of CEX are based on immunochemical and enzymatic assays. Nevertheless, the results were good, but disadvantages were shown by means of expertise and additional equipment requirement (*11*). Recently, an immunochromatographic assay for rapid multiresidue detection of cephems in milk was developed (*12*). However, the prepared polyclonal antibody showed a strong cross-reactivity with many cephems, and the cross-reactivity with other chemicals in milk was not described. There is a great need for a CEX detection method that is sensitive, specific, and easy in handling performance. Therefore, the objective of this study was (a) to produce a monoclonal antibody (mAb) for the application of CEX detection; (b) to test and evaluate this mAb with a one-step strip assay and ELISA for semiquantitative and qualitative CEX residue detection in milk and beef; and (c) validate and compare these results in relation to accuracy and practical handling.

MATERIALS AND METHODS

Chemicals and Supplies. Cephalexin hydrate, bovine serum albumin (BSA), ovalbumin (OVA) 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), goat antimouse IgG−horseradish peroxidase conjugate, *o*-phenylenediamine

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dihydrochloride (OPD), hydrogen peroxide, and Freund's adjuvants were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Antibiotic-Antimycotic, polyethylene glycol 1,500 (PEG 1,500), hyphoxanthine−aminopterine−thymidine (HAT) supplement, and hyphoxanthine−thymidine (HT) supplement were obtained from GIBCO BRL (Rockville, MD, USA). ImmunoPure (A/G) IgG Purification Kit was purchased form PIERCE (Rockford, IL, USA). BALB/c mice were obtained from Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (Beijing, China).

Apparatus. Cell culture plastic products and microtiter plates used for the ELISA analysis were obtained from GIBCO BRL. The plate reader for ELISA was from TECAN U.S, Inc. (Durham, NC, USA). Dispensing Platform ZX1000 and CM4000 Guillotine Cutting Module used to prepare test strips were purchased from BioDot Inc. (Irvine, CA, USA). MALDI-TOF/TOF proteomics analyzer 4700 was purchased from Applied Biosystem Inc. (Foster, CA, USA)

Preparation of Cephalexin Immunogen. Immunogen was prepared according to the procedure described by Paul P. Dillon et al. using EDC and NHS (*13*). The amine group of CEX was activated by EDC and linked with the carboxyl group of BSA. Briefly, CEX was dissolved in borate buffer (0.2 M, pH 8.5) to 10 mg/ml. EDC and NHS were then added to the final molarity of 0.4 and 0.1M, respectively. This mixture was then incubated at room temperature without agitation for 10 min. BSA solution (in borate buffer, molar ratio of BSA and CEX was 1:100) was added dropwise to the CEX solution. This solution was incubated at room temperature for 2 h and desalted by dialyzing against 1000 volumes of PBS (0.01 M, pH 7.4) overnight at 4 °C and stored at −70 °C until use. Avoidance of potential interference caused by antibodies against NHS and BSA, the CEX-OVA conjugate was prepared without using NHS.

Coupling efficiency of the CEX-BSA conjugate was determined with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (*14*). CEX-BSA solution was applied to Desalting Column (PIERCE, IL, USA) for desalting. BSA and CEX-BSA were diluted in distilled water to 1 mg/mL and mixed with an equivalent volume of matrix saturated solution. Matrix solution consisting of 10 mg/mL of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile water solution including 0.1% trifluoroacetic acid was prepared daily. Mixed solution was spotted directly on one well of the stainless MALDI plate and air-dried. The number of conjugated CEX molecules per BSA molecule was calculated from the mass difference of unconjugated to conjugated protein.

Monoclonal Antibody Production and Purification. Four 8 to 10 week old female BALB/c mice were immunized as described previously (*15*). Animals were handled according to the guidelines of the regional Animal Ethics Committee. All mice were injected intraperitoneally with 100 μg of CEX-BSA in Freund's complete adjuvant. Three booster shots were given at 2 week intervals with 100 μg of CEX-BSA in Freund's incomplete adjuvant. Blood was gathered 10 days after each injection, and the serum titers were tested by ELISA as described below to determine the antibody production. Four days prior to cell fusion, the mouse with the most sensitive CEX antibody in serum tested by ELISA was intraperitoneally given a final injection of 100 μg of CEX-BSA in PBS.

Splenocytes obtained from the immunized mice were fused with the myeloma cells SP2/0 using PEG 1,500. Hybrid cells secreting CEX specific antibody were selected by screening the culture supernatants using ELISA with CEX-OVA as coating antigen. The monoclonal cell line was isolated by twice subcloning with limiting dilution.

A large volume of antibody was obtained by in vivo approach. Hybridomas were intraperitoneally injected into adult BALB/c mice and the ascetic fluids were collected. Immunoglobulin was purified from the ascetic fluid of each mouse by ammonium sulfate precipitation. The precipitate was dissolved in 1 mL of PBS (0.01 M, pH 7.4) and applied to Desalting Column (PIERCE, IL, USA). Subsequently, the fractions of the high antibody titer were confirmed by ELISA, and the pooled fraction was applied to Protein A/G Column (PIERCE, IL, USA). After washing, the bound antibody was eluted from the column with 10 mL of elution buffer (0.1 M glycine-HCl, pH 2.5). Neutralization buffer (100 μL) was added to the collection tubes. Fractions of high antibody titer were confirmed by ELISA, and the pooled fraction was dialyzed in PBS for 12 h and then stored at −20 °C for subsequent tests.

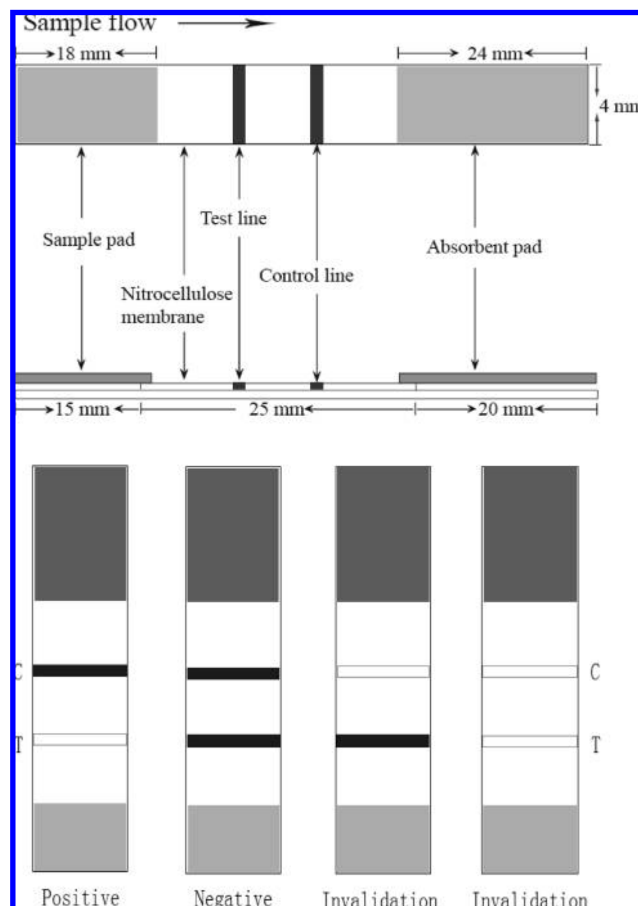

Figure 1. Schematic description of the colloidal gold-based one-step strip. The assay is based on a competitive reaction theory. The sample rapidly spreads through to the conjugate pad when it is applied to the sample pad. Then, mAb-Gs begin to migrate along with the sample flow. If CEX is absent in the test sample, the detection reagents (mAb-Gs) are trapped by the capture reagent (CEX-OVA) to form a visible test line, and the excessive mAb-Gs are bound with the capture reagent (goat antimouse IgG) in the control line. The red test line and control line are then developed. In contrast, if CEX is present in the sample, existing sufficient CEX molecules hinder the detection reagents (mAb-Gs) from combining with the capture reagent (CEX-OVA); thus, a positive sample gave no visible test line on the nitrocellulose membrane. When the test procedure is properly carried out, the control line is always visible. The result can be visualized by the naked eye, and the intensity of the test line is in proportion to the amount of CEX present in the samples.

Competitive ELISA. Each well of the microtiter plates was coated with 100 μL of CEX-OVA (1 μg/mL, in carbonate buffer, pH 9.6) and incubated for 2 h at 37 °C. Unbound CEX-OVA was removed from the plate with the wash buffer (PBST, 0.01 M, containing 0.1% Tween 20, pH 7.4), and each well was blocked with 200 μL blocking solution [4% (w/v) skim milk in carbonate buffer] at 37 °C for 1 h. Standards of CEX (50 μL each) ranging from 0.1 to 1000 μg/kg were added to each well and incubated with 50 μL of anti-CEX mAb (diluted 1/1000 in PBS) for 1 h at 37 °C. After removing the unbound CEX and antibody with the washing buffer, 100 μL of HRP labeled antimouse IgG was added (diluted 1/5000 in PBS) and incubated at 37 °C for 1 h. After washing three times, 100 μL of substrate solution [citrate buffer, containing 0.04% (w/v) OPD 0.01% (v/v) H_2O_2, pH 5.0] was added to each well and incubated for 20 min at 37 °C. Absorbance was measured at 490 nm using the ELISA reader after the addition of stop solution (3 M hydrochloric acid). Limit of detection was calculated as defined by the mean response obtained for the blank plus three standard deviations.

Cross-Reactivity with β-Lactams and Other Common Similar Chemicals. To determine the specificity of CEX antibody, cross-reactivity

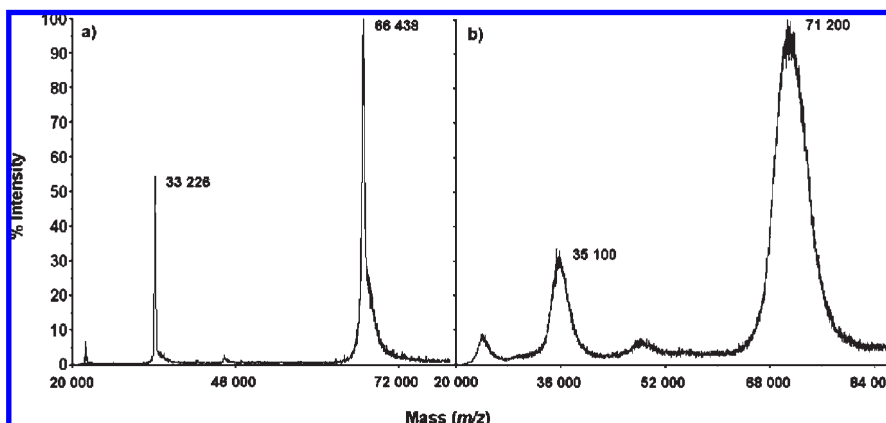

Figure 2. Mass/z of BSA and BSA-CEX. (**a**) Mass of BSA (66.44 kDa) and (**b**) the mass of the CEX-BSA conjugate (71.2 kDa). The calculated coating ratio of CEX and BSA is 14:1.

of the antibody with other β-lactams was determined by competitive indirect ELISA. Cefoperazone, ceftiofur, cefuroxime, cefaclor, sulfamethazine, and enrofloxacin were used as competitors. Cross-reactivities at a B/B_0 value of 50% (CR_{50}) were calculated by the method described previously (*16*), where OD values in the presence (B) and in the absence (B_0) of β-lactams were obtained.

One-Step Strip Preparation and Test Procedure. The colloidal gold (40 nm in diameter) preparation and strip assembly procedure was similar to that described by Jin et al. (*17*) and Wang et al. (*18*) with modifications. A schematic description of the colloidal gold-based one-step strip is illustrated in **Figure 1**. Gold-labeled anti-CEX mAb (mAb-G) was dispensed onto a conjugate nitrocellulose membrane pad (2 μL/cm, Millipore) and then dried for 1 h at 37 °C. Goat antimouse IgG (1 μg/kg) as the control line and CEX-OVA (0.65 μg/kg) as the test line were applied to the nitrocellulose membrane. Dispensed volumes were both 0.8 μL/cm per line. Four drops (about 200 μL) of standard solution (ranging from 0.1 to 100 μg/kg) or sample extract were diluted in Tris-HCl buffer. Consequently, 2 μL of colloidal gold-labeled mAb was added onto the sample pad, and the solution migrated toward the absorbent pad. Test line and control line were then developed depending on the concentration of CEX, results then can be visualized by the naked eye.

Monitoring of Cephalexin Concentration in Milk and Beef. Milk samples were collected from dairy farms, and CEX standards were added. Spiked milk samples and control were defatted by centrifuging at 15,000g at 20 °C for 10 min. Cephalexin was added into the obtained defatted middle layer in concentrations of 0, 5, 50, 100, 250, 500, 5000 μg/kg. Different starting amounts of CEX were diluted in 50-fold PBS, PBS (0.01M, pH 7.4) + Tween 20 [0.5%(v/v)], and PBS + BSA [0.5%(w/v)] + Tween buffers until the final concentrations of CEX (0, 0.1, 1, 2, 5, 10, 25, and 100 μg/kg) were obtained. Values of sample ODs via ELISA were calculated by the standard curve.

Beef samples obtained from a grocery store were homogenized, and CEX standards (0, 5, 50, 100, 250, 500, 5000 μg/kg) were added. Five grams of the samples was vortexed thoroughly with 10 mL of methanol for 5 min and then centrifuged at 15,000g at 20 °C for 10 min. The methanol layer containing CEX was filtrated with filter paper, vaporized, and consequently diluted in PBS + BSA + Tween buffer for the final concentrations of CEX (0, 0.1, 1, 2, 5, 10, 25, 100 μg/kg).

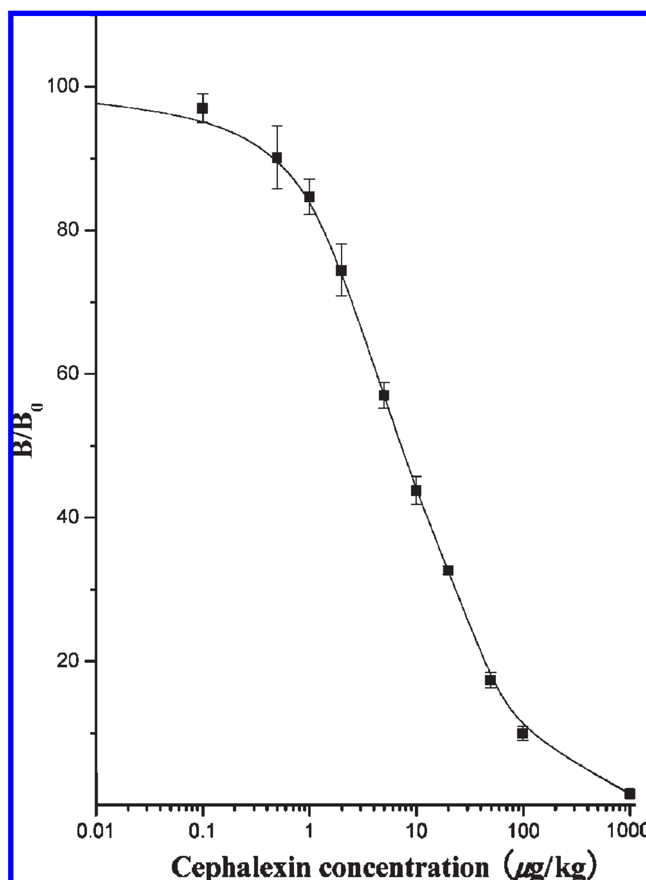

Figure 3. Standard competitive curve of CEX via competitive indirect ELISA in PBS. Absorbance of B/B_0 at 490 nm depends on CEX concentration. Each value shows the mean (±SD) of B/B_0 (n = 4). Limit of detection and IC_{50} are calculated by this curve.

RESULTS AND DISCUSSION

Preparation of CEX-BSA and CEX-OVA Conjugates. Cephalexin, like most other antibiotics, is a low-molecular weight organic compound and thus is devoid of any immunogenicity. Nevertheless, through the conjugation of CEX with a protein or polypeptide carrier, an antibody against the haptenic group can be obtained. Because of its high molecular weight (66 kDa) and a large number of functional groups, BSA is a well established carrier protein for the production of immunogen conjugate (*19*). To generate the CEX-specific antibody, the CEX-BSA conjugate was synthesized and used as an immunogen. Primary amines of CEX were conjugated with the carboxylic groups of BSA using EDC as a coupling reagent.

As the conjugate is crucial for the success of immunization, initial analysis of the conjugate was made with sodium dedecyl dulfate−poliyacrylamide gel electorphoresis (SDS−PAGE; figure not shown). Consequently, masses of the BSA and CEX-BSA conjugates were determined via MALDI-TOF MS (**Figure 2**). The conjugate coupling ratio (molar ratio) of CEX-BSA was calculated to be 14:1, which is optimal for further immunogen and coating antigen preparation (*20*).

Table 1. Intra- and Inter-Assay Validation of Cephalexin ELISA

	cephalexin concentration (μg/kg)									
parameter	0.1	0.5	1	2	5	10	20	50	100	1000
(average B/B_0 %)intra-assay	97.03	90.17	84.67	74.50	57.03	43.80	32.70	17.40	9.97	1.60
SD (%) (n = 4) standard deviation	1.99	4.40	2.47	3.58	1.76	1.93	0.53	1.08	0.95	0.44
variation coefficient (%)	2.05	4.89	2.92	4.80	3.09	4.40	1.62	6.22	9.48	27.24
(average B/B_0 %) inter-assay	96.43	90.08	85.27	75.15	54.85	40.03	32.80	14.56	7.01	1.70
SD (%) (n = 4) standard deviation	1.90	2.92	2.66	4.23	2.58	4.02	0.38	3.45	1.76	0.27
variation coefficient (%)	1.97	3.24	3.12	5.63	4.71	10.05	1.17	23.73	25.12	15.93

Table 2. Recoveries of Cephalexin from Spiked Milk and Beef (n = 7)

samples	level added [a](μg/kg)	level found [b] ± SD (μg/kg)	recovery (%)	CV (%)
milk	1.00	0.84 ± 0.18	84.00	14.29
	2.00	2.13 ± 0.17	106.50	7.98
	5.00	4.56 ± 0.27	91.20	5.92
	10.00	11.28 ± 1.13	112.80	10.02
beef	1.00	1.24 ± 0.31	124.00	25.00
	2.00	1.83 ± 0.16	91.50	8.74
	5.00	4.89 ± 0.83	97.80	16.97
	10.00	8.28 ± 0.43	82.80	4.88

[a] Prepared by dissolving CEX in bovine milk or extracted beef samples to give final concentrations diluted in PBS. [b] Calculated from the standard curves constructed by competitive indirect ELISA.

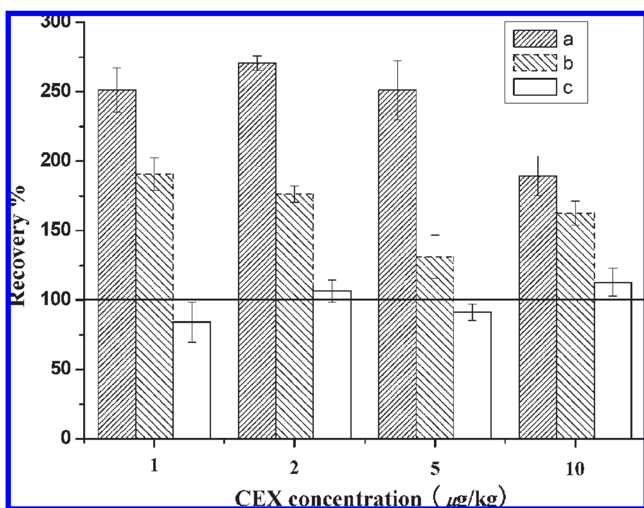

Figure 4. Assessment of ELISA matrix effects of CEX detection in milk. Standards and samples diluted in (**a**) PBS (0.01 M, pH 7.4) and (**b**) PBS with 0.5% Tween 20 showed unfavorable recoveries. Minimization of matrix effects were achieved with (**c**) added 0.5% Tween 20 and 0.5% BSA in PBS.

Cross-Reactivity with Other β-Lactams and Other Common Similar Chemicals. Cross-reactivity of the antibody with other β-lactams (cefoperazone, ceftiofur, cefuroxime, and cefaclor) and other common similar chemicals (sulfamethazine and enrofloxacin) was determined by competitive indirect ELISA as mentioned above. Calculated B/B_0 values of 50% (CR_{50}) did not show any cross-reactivity with applied chemicals in the observed concentration range of 1−10^4 μg/kg (data not shown).

Competitive Indirect ELISA. In a recent study, it was shown that the detection range of CEX was 3 to 30 μg/kg and that cross-reactivities were robust to interferences, but complicated performance and equipment were required (*11*).

To determine the detection limits of CEX in milk and beef, standard curves were constructed by competitive indirect ELISA (**Figure 3**). It showed a sigmoid shape for the CEX concentration range of 0−1000 μg/kg and was fitted by the five-parameter semilogarithmic model. Detection limit of 0.39 μg/kg in PBS was defined as the mean background level plus three times SD. The figure showed a detection range about 1−50 μg/kg, which indicates in comparison with recent studies, a normal accuracy level. Furthermore, there is a significant correlation in the quantitative measurable range of CEX with the concentration (r^2 = 0.99939; data not shown). On the basis of linearity, the level of accuracy can be mentioned as confidential in comparison with that in other studies (*11*, *12*).

Additionally, samples (n = 7) with added and excluded CEX were tested on trueness. Results showed no false-negative or false-positive samples (data not shown). Limit of detection for CEX is 0.39 μg/kg in PBS and resulting in 19.5 μg/kg in beef and milk by 50-fold dilution. This is below the MRL of the EU and shows similarities with recent studies (*20*, *21*). Intra- and interassay validations of CEX ELISA were evaluated as showed in **Table 1**.

Recoveries of CEX in spiked milk and beef with different concentrations (1, 2, 5, and 10 μg/kg) were determined by competitive ELISA, on the basis of the values obtained from the standard curve (**Table 2**). These results indicated that competitive ELISA was reliable to detect CEX in milk and beef even at low concentrations. Quantitative recoveries were in the range 84−124% for both milk and beef (**Table 2**).

Sample matrix, which usually contains protein and fat, might influence the antibody−antigen binding and other aspects of the immunoassays (*24*, *25*). Matrix effects decrease the OD values of ELISA and reduce the reliability and sensitivity as observed from the standard competitive curve (*18*). Therefore, different sample dilution buffers were used for the reduction of matrix effects (**Figure 4**). Hereby, optimized results were obtained with standard buffer PBS (0.01 M, pH 7.4) by the addition of Tween 20 [0.5% (v/v)] and BSA [0.5% (w/v)]. This is different from a recent study about reduced matrix effects in milk and beef samples where the results indicate optimized buffer dissolved with PBS and Tween 20 (*18*).

Immunochromatographic Strip Assay. As knowledge of assay specificity is crucial for correct data interpretation (*22*), a cross-reactivity test of gold-labeled mAb was performed. Distinguishable optical cross-reaction within applied chemicals is marginal (**Figure 5**). This proves that the specificity is accurate against CEX to detect negative samples as negative and stays in contrast to cross-reactivities with cephems using group-specific polyclonal antibody (*12*). To our knowledge, there are just a few publications concerning mAb for β-lactams, and they describe cross-reactivities (*6*). Even in comparison with mAb for antibiotics, in general our findings show an equivalent level of specificity (*7*, *8*).

Detection limits of the immunochromatographic assay were estimated at < 0.5 μg/kg of CEX in PBS (**Figure 6a**) and < 1 μg/kg in milk (**Figure 6b**) and beef (**Figure 6c**). Hereby, the control line

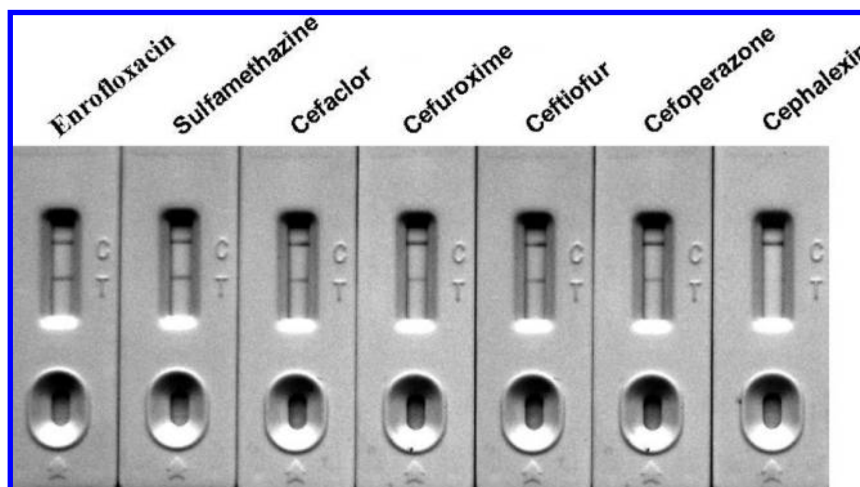

Figure 5. Cross-reaction of applied chemicals (50 μg/kg) in milk via the colloidal gold based one-step strip assay are optically identified.

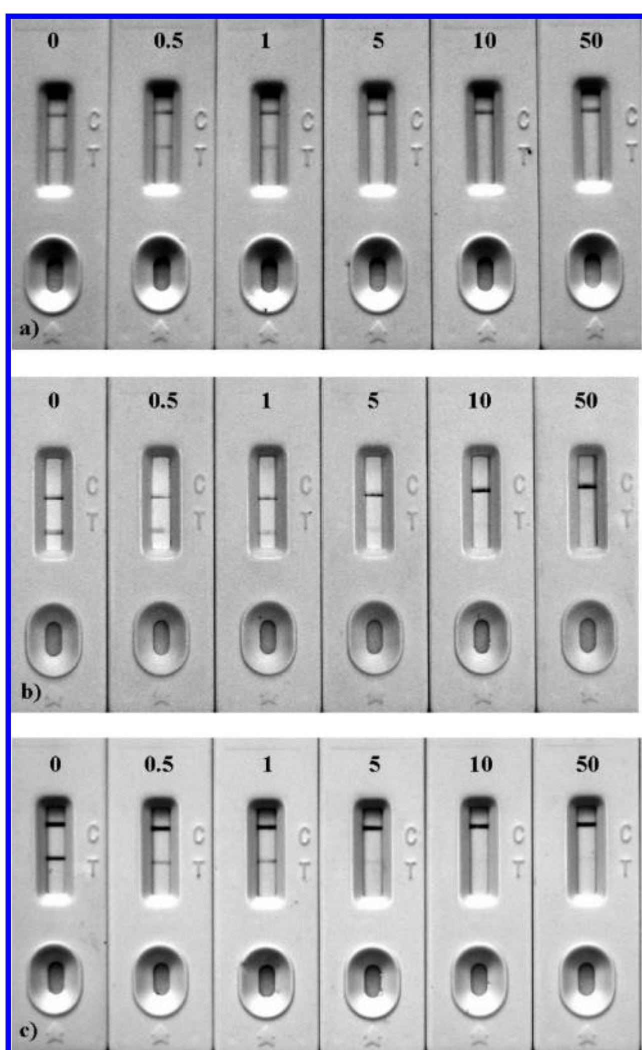

Figure 6. Results of CEX detection via the colloidal gold immunochromatographic strip assay (μg/kg). Detection limits are viewable (**a**) in PBS, (**b**) in spiked milk, and (**c**) in spiked beef.

showed maximum color development, which indicates a valid test (*23*). Color intensity gradually decreased with increasing concentrations of CEX and disappeared completely at 5 μg/kg in PBS (**Figure 6a**), 10 μg/kg in milk (**Figure 6b**), and 10 μg/kg in beef (**Figure 6c**), indicating a strong positive result. It is worth mentioning here that the decrease of quantitative color is subjective. Nevertheless, visual determination of absence and viewable line is affected by various parameters but shows that it gradually decreases over a certain concentration range (*22*). For practical on-site relevance and qualitative decisions, the distinguishable color intensity by the naked eye can be used.

In conclusion, because of its rapid and simple performance with high throughput and sensitivity, the colloidal gold-based one-step strip assay can be applied to the detection of CEX. The described colloidal-gold based one-strip assay with the proposed antibody is reliable and accurate for laboratory independent-on-site test to maximize consumers' security. However, more accurate detection should be enhanced by a more sensitive laboratory method such as the competitive indirect ELISA method. The assays developed in this study could complement each other and provide a useful tool for veterinary medicine. Moreover, instead of complicated equipment operation, such as HPLC, the methods developed in the present study can be applied to rapidly, conveniently, and economically determine CEX concentration in animal-origin food.

ABBREVIATIONS USED

ELISA, enzyme-linked immunosorbent assay; CEX, cephalexin; mAb, monoclonal antibody; CEX-BSA, Cephalexin−bovine serum albumin conjugate; CR_{50}, cross-reactivity; MRL, maximum residue limit; HPLC-MS, high performance liquid chromatography−tandem mass spectrometry; BSA, bovine serum albumin; OVA, ovalbumin egg; NHS, *N*-hydroxysuccinimide; EDC, 1-ethyl−3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride; IgG, Immunoglobulin G; HRP, horseradish peroxidase conjugate; OPD, *o*-phenylenediamine dihydrochloride; DMEM, Dulbecco's modified Eagle's medium; HAT, hyphoxanthine−aminopterine−thymidine; HT, hyphoxanthine−thymidine; FBS, fetal bovine serum; mAb-Gs, monoclonal antibody labeled gold.

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