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Preparation of Anti-Tetracycline Antibodies and Development of an Indirect Heterologous Competitive Enzyme-Linked Immunosorbent Assay to Detect Residues of Tetracycline in Milk

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Tetracycline (TC) is a broad-spectrum antibiotic used increasingly in animal husbandry to treat diseases or to promote growth as feed additives. To avoid using labor-intensive instrumental methods to detect residues of TC in food and food products, a simple and convenient indirect heterologous competitive enzyme-linked immunosorbent assay (ELISA) method for TC was developed using polyclonal antibody prepared in this study. Three new immunogens, TC-*o*-tolidine-bovine serum albumin (BSA), TC-4-aminobenzoic acid-cationized BSA (cBSA), and TC-1,1′-carbonyldiimidazole-cBSA, were synthesized in this research to develop anti-TC antibodies. All antibodies raised in rabbits and coating antigens synthesized were screened and characterized using homologous and heterologous ELISA formats to select the best combination. An optimized ELISA gave an IC_{50} value of 3.92 μg/mL toward TC in PBS buffer. The specificity of the assay was studied by measuring cross-reactivity of the antibody with the structurally closely related compounds of chlortetracycline (112%) and oxytetracycline (<2%). The recovery rates from the TC-fortified raw milk samples were in the range of 74−116%, while the intra- and interassay coefficients of variation were <14.5 and <25.0, respectively.

KEYWORDS: Tetracycline; ELISA; drug residue; antibody; immunoassay

INTRODUCTION

Tetracyclines (TCs; **Figure 1**) are a group of broad-spectrum antibiotics used for medical purposes as well as animal husbandry (*1*). The TC antibiotics have a broad range of activity against a variety of both Gram-positive and Gram-negative bacteria. Furthermore, they are both easy to administer, effective through oral dosing via water and feed, and inexpensive (*1*−*3*). For these reasons, the TC antibiotics are extremely popular as veterinary antibiotics in animal husbandry either for the prevention and treatment of diseases or for feed additives to promote growth. The TC antibiotics are licensed for use in a variety of food-producing animals including cattle, pig, poultry, and fish (*4*, *5*). In 1998, the European Federation of Animal Health carried out a survey on the veterinary use of antibiotics in the 15 member States of the European Union (EU) and Switzerland. It was found that TC antibiotics accounted for 65% of all antibiotics and antibacterials consumed for therapeutic and preventive use (*4*).

The use of TC antibiotics in animal husbandry, however, has the potential to result in the presence of residues in tissues and the increased emergence of resistant strains of pathogenic bacteria that could have potential health risks to humans (*5*, *6*). Nowadays, antibiotic resistance has become a global threat because existing antibiotics are becoming increasingly ineffective in combating microbial infections in humans (*6*). To ensure the safety of food for consumers, more and more countries have set MRLs (maximum residue levels) and withdrawal periods

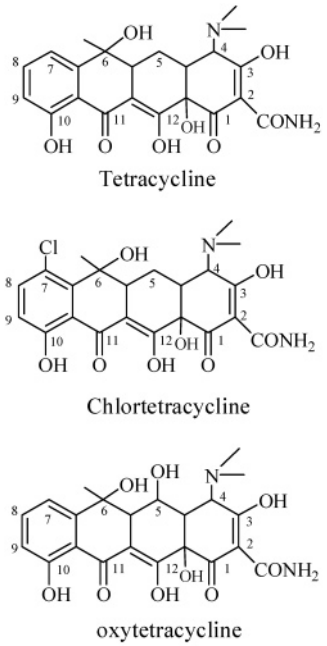

Figure 1. Chemical structures of TC and related compounds evaluated in this study.

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for TC antibiotics. In the United States, the MRLs of TC are 2 ppm in muscle, 6 ppm in liver, and 12 ppm in kidney (*7*).

In China, a withdrawal period of at least 4 days is required before livestock can be slaughtered for food purposes (no. 278, 2003.5.22). To detect residues of TCs, suitable analytical techniques have to be established. The microbiological assays are usually used for the measurement of TCs in food because they are easy to perform and inexpensive. However, these methods are complicated, time-consuming, and lack specificity (*8*). Instrumental analyses such as liquid chromatography−mass spectrometry (LC-MS) (*4*, *6*) and high-performance liquid chromatography (HPLC) (*9*, *10*) are the most widely used methods to detect residues of TC in food and food products.

These methods are sensitive and highly specific but require expensive equipment, large volume of solvents, derivatizing treatment, and time-consuming sample cleanup process. Therefore, they are not suitable to be used as routing screens and field detection for TCs.

The enzyme-linked immunosorbent assay (ELISA) technique has long been known as a rapid, sensitive, specific, and cost-effective analytical method and has been used for diagnostic and residue detection purposes for many years. The first step to develop a high quality ELISA test kit to detect residues of TC is to prepare a high-qualified immunogen of TC. TC is an unstable substance under acidic and basic conditions and is sensitive to humidity and heat. Besides, its complex chemical structure also makes it difficult to synthesize qualified immunogens.

There are no reports concerning the synthesis of TC immunogen and preparation of anti-TC antibody so far in literature, although commercially the ELISA kits to detect residues of TC are available from Biopharm (Darmstadt, Germany). This paper disclosed the detailed information related to synthesis of TC immuogens and characterization of corresponding antibodies. The ELISA procedure based on the antibody prepared was developed and used to detect TC residues in milk.

MATERIALS AND METHODS

Chemicals and Materials. TC, *o*-tolidine (tolidine), bovine serum albumin (BSA), ovalbumin (OVA), 1-ethyl-3-(dimethylaminopropyl)-carbodiimide hydrochloride (EDC), 4-aminobenzoic acid (ABA), 1,1′-carbonyldiimidazole (CDI), and Freund's complete and incomplete adjuvants (cFA and iFA) were purchased from Sigma-Aldrich (St. Louis, MO). *N*-Hydroxysuccinimide (NHS) was provided by Cxbio Biotechnology Ltd. (Shanghai, China).

3,3′,5,5′-Tetramethylbenzidine (TMB) was purchased from Amresco (Solon, OH). Goat anti-rabbit IgG-horseradish peroxidase conjugate was provided by the Military Medical Institute (Beijing, China). *o*-Phenylenediamine (OPD) was purchased from Xinjingke Biotechnology (Beijing, China). Acetone was HPLC grade obtained from Tianjin Kermel Chemical Reagents Development Center (Tianjin, China). Ethylenediamine dihydrochloride (EDA), hydrogen peroxide (30%), and other reagents used were chemical grade from Guangmang Chemical Co. (Jinan, China).

Instrumentation and Supplies. ELISA was performed in polystyrene 96 well microtiter plates (Bio Basic Inc.) and spectrophotometrically read with an automatic microplate reader KHB ST-360 from Shanghai Zhihua Medical Instrument Ltd. UV data were collected on a U-4100 spectrophotometer from Hitachi Co. Centrifugation was carried out with a refrigerated centrifuge (Biofuge Stratos, Heraeus). Protein dialyses were performed using dialysis tubes from Aibo Economic & Trade Co., Ltd. (Jinan, China). Rotary evaporation was carried out with the rotary evaporator (RE-5203A) from Shanghai Zhenjie Laboratory Instrument Co., Ltd.

Buffers. For the preparation of all buffers and reagents for the immunoassays, ultrapure deionized water was used. Phosphate-buffered saline (PBS, pH 7.4) consisted of 138 mM NaCl, 1.5 mM KH_2PO_4, 7 mM Na_2HPO_4, and 2.7 mM KCl. The wash buffer (PBST) was a PBS buffer containing 0.05% Tween 20. Sodium borate buffer (pH 8.5) consisted of 150 mM NaCl and 500 mM sodium borate. As a coating buffer, 0.05 M carbonate buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6) was used. The blocking buffer was PBS + 1% OVA + 0.05% (v/v) Tween 20. The substrate buffer was 0.1 M sodium acetate/citrate buffer (pH 5.0). To prepare the substrate solution, 10 mg of OPD was dissolved in 25 mL of sodium citrate buffer and this solution plus 5 μL of H_2O_2 [30% (w/w)]. The stopping solution was 2 N HCl.

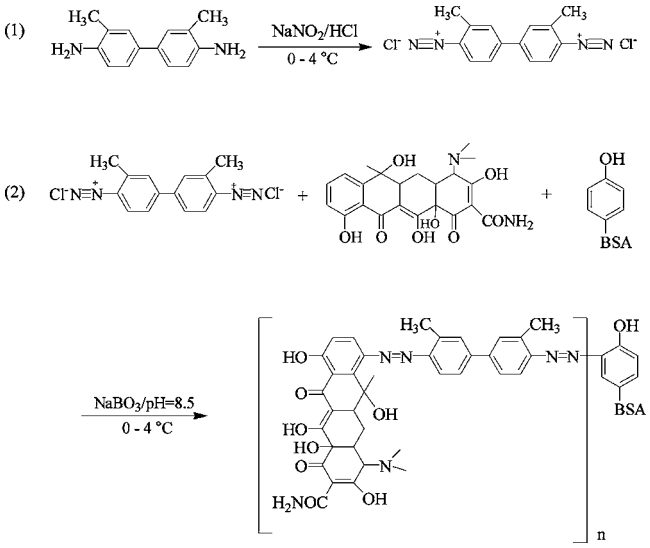

Figure 2. Synthetic procedure for TC immunogen of TC-tolidine-BSA through the tolidine method.

Preparation of Cationized BSA (cBSA) and Cationized OVA (cOVA). In this procedure, carboxylic acid groups of the carrier proteins of BSA and OVA were converted into primary amine groups with an excess of EDA. A solution of 1 g of BSA (15 μmol) and 56 mg of EDC (300 μmol) in 20 mL of PBS (0.01 M, pH 7.4) was added slowly into a solution of 18 mg of EDA (300 μmol) in 20 mL of PBS (0.01 M, pH 7.4) under stirring. The mixture solution was incubated continuously for 2 h at room temperature and then dialyzed [molecular weight cutoff (mwco), 12000−14000 Da] under stirring against PBS (0.01 M, pH 7.4) to remove free EDA. Cationized BSA and OVA were defined as cBSA and cOVA, respectively. The solution was lyophilized, and the white solid (cBSA) obtained was stored at −20 °C before it was used in the next reaction. The cOVA was prepared in a similar method.

Preparation of Immunogens and Coating Antigens. *Tolidine Method (TC-Tolidine-BSA and TC-Tolidine-OVA).* The immunogen TC-tolidine-BSA and the coating antigen TC-tolidine-OVA were prepared by the homobifunctional method as described in the literature (*11*). In this procedure (**Figure 2**), 25 mg of tolidine (118 μmol) was dissolved in 4.5 mL of 0.2 N HCl followed by the dropwise addition of 17.5 mg of sodium nitrite (254 μmol) in 0.5 mL of distilled water in the dark at 4 °C with constant stirring. The reaction mixture was allowed to stand for 30 min. Subsequently, 1.0 mL of orange bis-diazotized tolidine solution was slowly added to 2 mL of 0.5 M sodium borate solution (pH 8.5, containing 0.15 M NaCl) containing 20 mg of BSA (0.294 μmol) and 10 mg of TC (22.5 μmol). The color of the mixture solution immediately changed into purple. The reaction was incubated for 2 h at 4 °C in the dark.

The purple reaction mixture was dialyzed (mwco, 12000−14000 Da) under stirring against PBS (0.01 M, pH 7.4) for 3 days with frequent changes of the PBS solution to remove the uncoupled free hapten. The precipitate was removed by centrifugation at 3000*g*, and the supernatant was lyophilized to obtain a purple conjugate of TC-tolidine-BSA, which was stored at −20 °C for future use. A conjugate of TC-tolidine-OVA was prepared in a similar method. An UV absorbance spectrum was employed to determine whether the linking had been a success (**Figure 3**).

ABA Method (TC-ABA-cBSA and TC-ABA-cOVA). In this procedure (**Figure 4**), 20 mg of ABA (145 μmol) was dissolved in 2.2 mL of 0.2

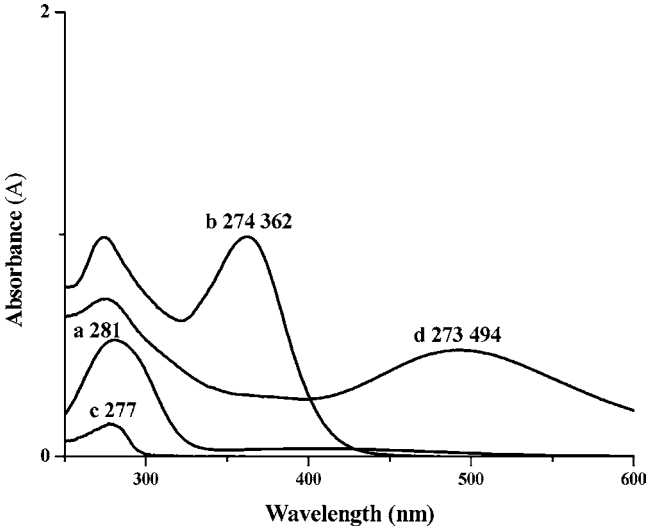

Figure 3. UV absorbance: (**a**) tolidine, (**b**) TC, (**c**) BSA, and (**d**) TC-tolidine-BSA.

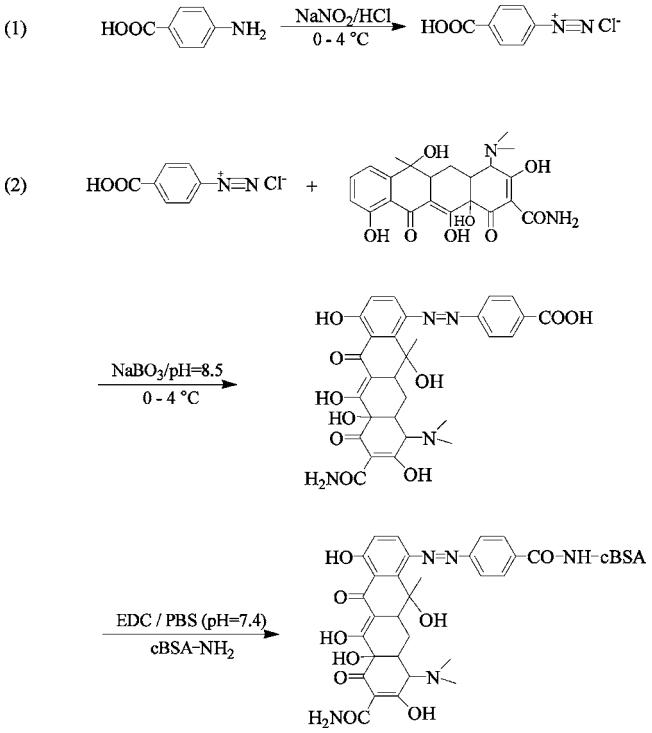

Figure 4. Synthetic procedure for TC immunogen of TC-ABA-cBSA through the ABA method.

N HCl followed by the dropwise addition of 12 mg of sodium nitrite (174 μmol) in 0.35 mL of distilled water in the dark at 4 °C with constant stirring. The reaction mixture was allowed to stand for 1 h. Subsequently, 1.5 mL of diazotized ABA solution was slowly added to 10 mL of 0.5 M sodium borate (pH 8.5, containing 0.15 M NaCl) containing 27 mg of TC (61 μmol). The mixture solution immediately became purple. The reaction was incubated for 2 h at 4 °C in the dark followed by addition of H_3BO_3 to adjust the pH to 7.4.

Subsequently, 136 mg of cBSA (2 μmol), 120 mg of EDC (626 μmol), and 36 mg of NHS (313 μmol) were added to the solution and the reaction was allowed to stand at room temperature for 3 h with constant stirring. The reaction mixture was dialyzed (mwco, 12000−14000 Da) under stirring against PBS (0.01 M, pH 7.4) for 3 days with frequent changes of the PBS solution to remove the uncoupled free hapten. The solution was lyophilized, and the purple TC-ABA-cBSA conjugate obtained was stored at −20 °C for future use. A TC-

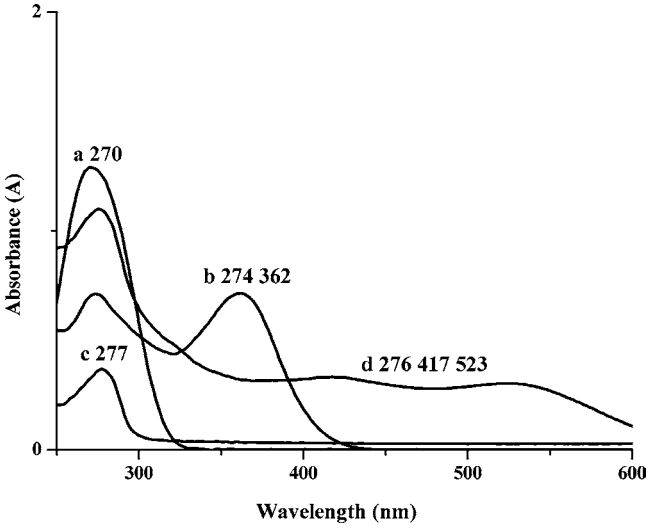

Figure 5. UV absorbance: (**a**) ABA, (**b**) TC, (**c**) cBSA, and (**d**) TC-ABA-cBSA.

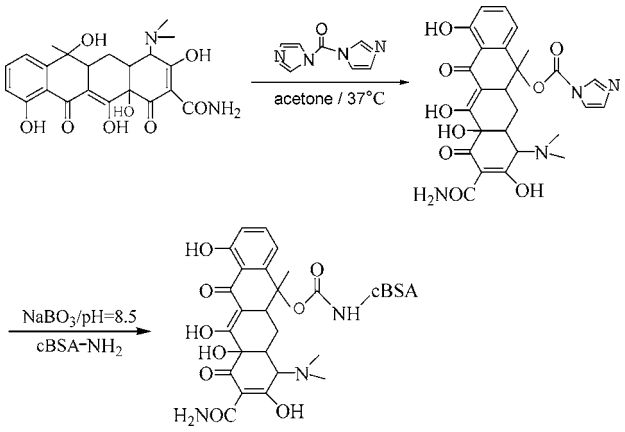

Figure 6. Synthetic procedure for TC immunogen of TC-CDI-cBSA through the CDI method.

ABA-cOVA conjugate was prepared in a similar method. An UV absorbance spectrum was employed to determine the coupling result (**Figure 5**).

CDI Method (TC-CDI-cBSA and TC-CDI-cOVA). In this procedure (**Figure 6**), 40 mg of TC (90 μmol) was dissolved in 8 mL of dry acetone. Subsequently, 29 mg of CDI (180 μmol) was quickly added to the solution. The reaction mixture was allowed to stand at 37 °C for 3 h in the dark under dry nitrogen. Acetone was evaporated using a rotary evaporator under vacuum, and the residue was redissolved in 15 mL of sodium borate (0.5 M, pH 8.5). To this solution, 200 mg of cBSA (3 μmol) was added, followed by incubation at room temperature for 48 h in the dark under dry nitrogen. The reaction mixture was dialyzed (mwco, 12000−14000 Da) under stirring against PBS (0.01 M, pH 7.4) for 3 days with frequent changes of the PBS solution to remove the uncoupled free hapten. The solution was lyophilized, and the yellow TC-CDI-cBSA conjugate obtained was stored at −20 °C for future use. A TC-CDI-cOVA conjugate was prepared in a similar method. An UV absorbance method was employed to determine whether the linking had been a success (**Figure 7**).

Immunization of Rabbits. Three immunogens of TC-tolidine-BSA, TC-ABA-cBSA, and TC-CDI-cBSA were used to immunize rabbits to prepare corresponding anti-TC antisera. For each immunogen, two male New Zealand white rabbits were subcutaneously immunized at multiple sites in the back. The initial immunization was subcutaneously injected with 0.5 mg of conjugate in 0.5 mL of NaCl (0.9%) and 0.5 mL of Freund's complete adjuvant. Subsequent booster injections [0.25 mg of conjugate in 0.5 mL of NaCl (0.9%) plus 0.5 mL of Freund's incomplete adjuvant] were performed 15 days later and then at 14 day intervals. One week after each booster, serum titers were tested by

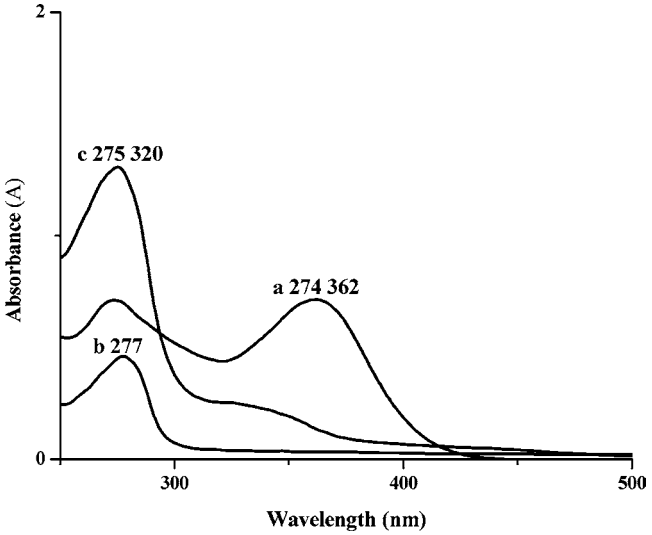

Figure 7. UV absorbance: (**a**) TC, (**b**) cBSA, and (**c**) TC-CDI-cBSA.

Table 1. Summary of Titers[a] of Antisera vs Three Coating Antigens

immunogens used to raise antibodies	coating antigens		
	TC-tolidine-OVA	TC-ABA-cOVA	TC-CDI-cOVA
TC-tolidine-BSA	1:16000	1:3200	1:3200
TC-ABA-cBSA	1:200	1:64000	1:200
TC-CDI-cBSA	1:1600	1:51200	1:512000

[a] The titer of antiserum is defined as the antiserum dilution that gave 2.0 times absorance of the control serum.

ELISA. The antiserum obtained after each booster was prepared by allowing the blood to clot overnight at 4 °C, followed by centrifugation to remove particulate material. Ten days after the last boost, all rabbits were then exsanguinated by heart puncture under general anesthetic and euthanized by lethal injection before recovery. The serum was separated from blood cells by storage of the blood overnight at 4 °C and centrifugation with 13000 rpm/min for 20 min. The crude serum obtained was purified by saturated ammonium sulfate (SAS) precipitation method [purified three times using 50, 33, and 33% (v/v) of SAS, respectively], and sodium azide was added as a preservative at a final concentration of 0.02% (w/w). The purified serum was then aliquotted and stored at −70 °C.

Antisera Evaluation. *Antibody Titer Determination by Indirect ELISA.* The titer of the serum from each animal was determined by measuring the binding of serial dilutions of the antisera to microplates with each of the three different coating antigens (TC-tolidine-OVA, TC-ABA-cOVA, and TC-CDI-cOVA).

The results are summarized in **Table 1** and the detailed processes are described below. Microplates were coated with three different coating antigens (10 μg/mL, 100 μL/well) in PBS (0.01 M, pH 7.4) by overnight incubation at 4 °C. Plates were washed with PBST three times and blocked with 250 μL/well of blocking buffer, followed by incubation for 2 h at room temperature. Plates were washed for three times again, the appropriate dilution of the antisera was added, and the plates were incubated for 2 h at room temperature. After another washing step, 100 μL/well of a diluted (1:1000) goat anti-rabbit IgG-HRP conjugate was added, followed by incubation for 1 h at room temperature. Then, plates were washed three times, and 100 μL/well of OPD substrate solution was added. The color development was halted by adding stopping solution (2 N HCl, 100 μL/well) after 15 min of incubation at room temperature, and absorbance was read at 492 nm. Absorbances were corrected by blank reading. Preimmune withdrew serum was used as a negative control, and the antibody titer was defined as the reciprocal of the dilution that resulted in an absorbance value that was twice that of the background.

Development of Indirect cELISA. The checker board procedure was used to optimize the coating antigen and the primary antibody concentrations. Three different coating antigens (TC-tolidine-OVA, TC-ABA-cOVA, and TC−CDI-cOVA) were used in a competitive ELISA for assessing the specificity of the serum from each animal to free TC.

To each well of a 96 well plate, 100 μL of 10 μg/mL of selected coating antigen solution in bicarbonate buffer (0.05 M, pH 9.6) was added and incubated overnight at 4 °C. The plate was washed with wash buffer three times and blocked with 250 μL/well of blocking buffer, followed by incubation for 1 h at room temperature. After the blocking solution was removed and the plate was washed three times, 100 ng of primary antibody was added to each well followed by the addition of buffer or competitor in buffer, and the plate was incubated for 2 h. The plate was washed three times, and goat antirabbit IgG-HRP (1:1000, 100 μL/well) was added, followed by incubation for 2 h at room temperature. The plate was washed three times, OPD substrate solution was added (50 μL/well), and the plate was incubated for another 30 min at room temperature. The color development was halted by adding stopping solution (50 μL/well), and absorbances were measured at 492 nm. Absorbances were corrected by blank reading. Preimmune withdrawn serum was used as a negative control. The result was expressed in percent inhibition as follows: % inhibition = $\%B/B_0$, where B is the absorbance of the well containing competitor and B_0 is the absorbance of the well without competitor.

Specificity Determination. After coating antigens were optimized by the above indirect competitive ELISA, the TC-ABA-cOVA was selected as a coating antigen to determine cross-reactivity and establish the standard calibration curve. Competitive immunoassays were performed using various compounds structurally related to TC, to determine the respective IC_{50} value and cross-reactivity. ELISA plates were coated with coating antigen at 10 μg/mL (100 μL/well) by incubation overnight at 4 °C and then washed three times. Plates were blocked (250 μL/well) for 2 h at room temperature and washed three times again. For the competition step, 50 μL/well of competitors (TC, chlortetracycline, and oxytetracycline) were added at a concentration ranging from 0.01 to 200 μg/mL and coincubated with 50 μL/well of primary antibody for 2 h. The secondary antibody and color development were the same as described above. After reading the plate, the IC_{50} value was determined by using the concentration of inhibitor that leads to a 50% decrease of the maximum signal; the cross-reactivity (%) was calculated as: $(IC_{50,TC})/(IC_{50,compound}) \times 100$ (*12*).

Standard Curve Generation. The TC-ABA-cOVA (10 μg/mL) was used as a coating antigen, and indirect competitive ELISA was performed as described above. The selected antisera at 1:1000 dilution were utilized as primary antibody and coincubated with TC. The standard calibration curve with final TC concentrations of 0.2, 0.5, 1.0, 2.0, 5.0, and 10 μg/mL was run in PBST.

Matrix Effects Determination. The milk sample was defatted by centrifugation at 4 °C (10000*g*, 15 min) and was artificially contaminated by adding drug standard solution in PBST. Competitive curves with final TC concentrations of 0.01, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10, and 20 μg/mL were run in PBST and in various dilutions (1:2, 1:10, and 1:20) of the defatted milk with PBST in order to determine the matrix effect of milk. IC_{50} and B_0 values from each diluted curve were obtained by comparing with IC_{50} and B_0 values generated from the PBST buffer.

Inter- and Intra-assay Variation Determination. The milk samples were fortified by TC at final concentrations of 0.2, 0.5, 1.0, and 2.0 μg /mL in milk diluted at 1:20 with PBST buffer. Interassay variation was computed from the analysis of four replicates of each dilution carried out on four different days. Intra-assay variation was measured by analysis of five replicates of each dilution on a single day. Sample recoveries were determined from a standard curve.

RESULTS AND DISCUSSION

Hapten Conjugation. As a small molecule with a molecular mass of 444.4, TC is not able to elicit the immune response of an animal to produce the anti-TC antibody and is, therefore, nonimmunogenic. To make it immunogenic, it must be conjugated to a carrier protein before immunization. Among protein

carriers, BSA and OVA are two of the most commonly used ones, and usually, they give satisfying results. BSA was treated with an excess of ethylenediamine (EDA) as described previously (*11*, *13*) to convert carboxylic acid groups into primary amine groups to prepare cBSA. The cBSA prepared has the advantage over BSA that more primary amino groups become available on cBSA to couple with functional groups, such as carboxylic groups, on hapten. Moreover, the use of cationized carrier proteins can minimize cross-linking and increase their pI values to generate more immune responses as compared to their native forms (*11*).

TC is quite stable in the dry state under normal storage conditions, but high temperature and humidity both facilitate TC degradation. TC changes color from light yellow to brown and dark brown after exposure to the humidity or heat. Moreover, TC is unstable in aqueous solution, especially in strong acid and strong alkaline solution. In the acid solution, because of the relative instability of hydroxyl group in C6 (see **Figure 1** for numbering of TC), strong acids dehydrate the C-ring through the hydroxyl group in C6 in TC molecule, forming 5-hydroxyanhydrotetracyclines. Furthermore, the dimethylamino group in C4 can undergo a reversible epimerization process in vitro and in vivo, producing a set of epimers with different antibacterial activities (*5*, *14*, *15*). In the alkaline solution, the C6 hydroxyl group easily loses the hydrogen, forming an oxide. The oxide can attack the C11 carbon, forming a lactone so that TC loses activity (*16*). On account of these reasons, the preparation of TC immunogen with a high quality was very difficult. To the best of our knowledge, the synthesis of TC immunogen for the purpose to prepare anti-TC antibody has not yet been reported in literature.

In this research, three conjugates of TC with carrier proteins were prepared. The first conjugate (TC-tolidine-BSA) was synthesized using tolidine, a homobifunctional cross-linking agent, as a bridge to link TC and carrier protein BSA by a simple one-step conjugation reaction. The two amino groups in tolidine were diazotized to create the requisite bis-diazonium derivative and then coupled simultaneously the para position of the aromatic hydroxyl group of TC with the ortho position of tyrosine residue in BSA, forming azo derivative (**Figure 2**). The diazo reaction proceeded by electrophilic attack of the diazonium group toward the electron-rich points on the target molecules (*11*). It is well-known that phenolic compounds are modified at positions ortho and para to the aromatic hydroxyl group. However, as far as electron effects and steric effects are concerned, the diazo reaction usually proceeds at the para position of the aromatic hydroxyl group. When the para position of the aromatic hydroxyl group has a substituent, the reaction conducts at the ortho position (*17*). Both the para and the ortho positions of the aromatic hydroxyl group of TC have no substituent; thus, the diazo reaction is more likely to proceed at the para position of TC according to the reason mentioned above. For tyrosine side chains of BSA, only the ortho modification is possible (**Figure 2**). To obtain evidence of successful conjugation, UV absorbances recorded from 250 to 600 nm were measured for BSA, TC, tolidine, and TC-tolidine-BSA conjugate as shown in **Figure 3**. BSA has an absorbance peak at 277 nm coming from an aromatic group in the molecule. Tolidine has one peak at 281 nm, a red shift as compared with BSA's 277 nm, because it has a longer conjugate system consisting of two benzene rings. In the conjugate of TC-tolidine-BSA, the conjugation system has been expended significantly because tolidine, consisting of two benzene rings itself, links two aromatic systems together to form the conjugate. The two nitrogen double bonds, working as two bridges in TC-tolidine-BSA, connect two small conjugation systems together to form a larger one (**Figure 2**). Consequently, we find a peak at a long wavelength of 494 nm in UV spectrum for TC-tolidine-BSA as the consequence of red shift in longer conjugation system (**Figure 3**) as expected. The corresponding coating antigen TC-tolidine-OVA gives a similar pattern in UV spectrometry.

In the conjugation reaction to synthesize the second TC conjugate TC-ABA-cBSA, a linking agent ABA was diazotized and linked with TC to introduce a carboxylic acid to form a modified TC. The cBSA, then, was connected with the modified TC to form a conjugate of TC-ABA-cBSA as shown in **Figure 4**. The carbodiimide of EDC was applied in the synthetic process to activate the reaction. The UV spectra recorded from 250 to 600 nm for TC, cBSA, ABA, and TC-ABA-cBSA were shown in **Figure 5**. An azo bond has been formed between TC and ABA, which increase the length of the conjugation system in TC-ABA-cBSA. The red shift in UV absorbance was observed for the conjugate (417, 523 nm) as expected. The coating antigen TC-ABA-OVA gives a similar UV pattern.

The third conjugate was synthesized using CDI as a linking and activation agent. In the alkaline solution, because the hydroxyl group in C6 is easy to lose the hydrogen to form the oxide, we believe that the reaction should occur at this hydroxyl group in the CDI method. The hydroxyl group in C6 as a nucleophile attacks the carbonyl carbon of CDI, forming an active imidazolyl carbamate. The imidazolyl carbamate is attacked by the amine-containing cBSA, forming the conjugate TC-CDI-cBSA. The synthetic pathway of this method is shown in **Figure 6**, and the UV spectra of TC, cBSA, and TC-CDI-cBSA, recorded from 250 to 500 nm, are shown in **Figure 7**. It can be seen that the cBSA has a peak at 277 nm, and TC has two peaks at 274 and 362 nm. The presence of carbonyl group between TC and cBSA made the density of the electron cloud of the conjugated system of TC fall, so that the requisite energy of electron transition increased.

Thus, the blue shift occurred in UV spectrometry as expected. A peak of TC at 362 nm had shifted to 320 nm of TC-CDI-cBSA in UV spectrometry. The coating antigen TC-CDI-cOVA gives a similar UV pattern.

Titers of the Antisera. The final antisera obtained from each rabbit were purified with SAS and used for antibody characterization. The titer of antibody from each rabbit was estimated by the indirect ELISA using either homologous or heterologous assays. Titers of the individual animals injected with the same immunogen showed little differences. Most antisera showed fairly high titers to their homologous antigens as compared to heterologous antigens. The selected representative examples are listed in **Table 1**. The results show that the antisera of number 6 rabbit from immunogen of TC-CDI-cBSA when using TC-ABA-cOVA as coating antigen give the best combinational results in terms of both affinity and specificity. We are going to use this combination throughout the remainder of this report. **Table 1** suggests that all of the antisera obtained, more or less, can distinguish the TC skeleton from hapten-protein conjugates.

Indirect Competitive ELISA: Recognition of Free TC. The inhibition in an indirect cELISA is affected by the strength of interaction between the antisera and the coating antigen. It is well-known that heterologous assays often help improve the immunoassay sensitivity (*18*−*21*) and overcome unwanted cross-reactivity associated with the strong affinity of the antibodies to the spacer arm that results in no or poor inhibition by the analyte (*22*, *23*). Heterology modifies equilibrium conditions between coating antigens and analyte so that stronger

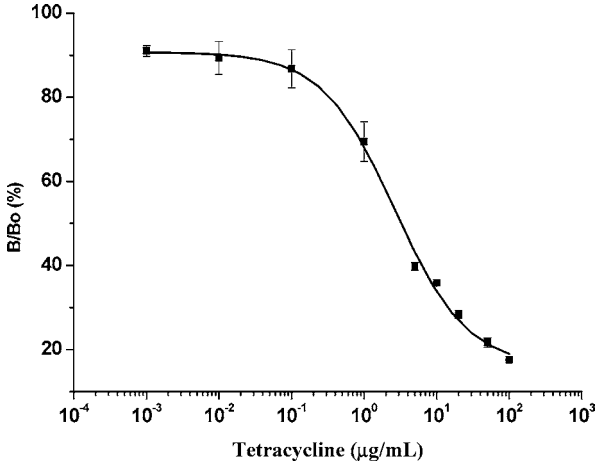

Figure 8. Representative inhibition curve of anti-TC antibody using TC as the competitor and TC-ABA-cOVA as the coating antigen in PBS buffer solution. Each point represents the average of five replicates.

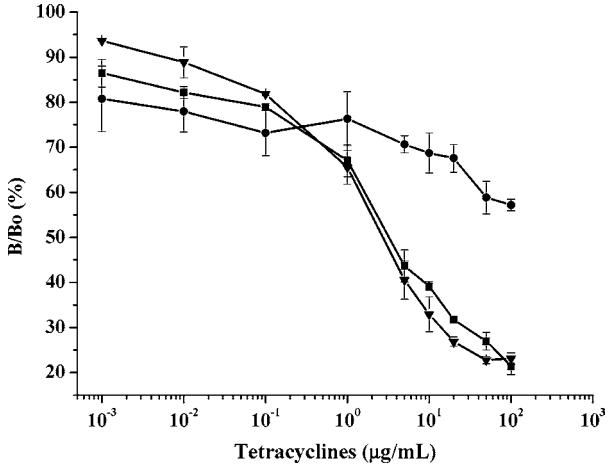

Figure 9. ELISA curve for polyclonal anti-TC antibody using TC (■), chlortetracycline (▼), and oxytetracycline (●) as competitors in PBS buffer solution. Each point represents the average of five replicates.

Table 2. IC_{50} and Percentage of Cross-Reactivity of TC Analogues

compound	IC_{50}[a]	cross-reactivity[b] (%)
TC	3.92	100
chlortetracycline	3.49	112
oxytetracycline	<200	<2

[a] IC_{50} values are in μg/mL. [b] Cross-reactivity was determined by comparing the concentration of analyte required to produce a $B/B_0 = 50\%$. Results were expressed as a percentage relative to the figure for TC.

recognition toward the analyte can be achieved (*24*, *25*). Suitable heterology can be carried out at different levels by hapten (use of partial structure or change of key determinants), site, linker, and spacer modification (composition, length, and conjugation chemistry) (*18*).

In view of the above-mentioned reasons, in this study, in order to set up a sensitive ELISA, all possible combinations between coating antigens and antibodies were screened via the indirect cELISA by serial concentrations (ranging from 0.01 to 100 μg/mL) of TC dissolved in PBST, using the homologous or heterologous assay. It was found that there was very low inhibition by TC in homologous assays when an immunogen and a coating antigen were prepared using a same linker.

In the heterologous assays, the combinations of TC-ABA-cOVA or TC-tolidine-OVA as coating antigens with the antibodies raised against immungen of TC-CDI-cBSA yielded the best results in inhibitions, particularly the combination between the TC-ABA-cOVA and the antibodies from rabbit number 6. **Figure 8** shows a TC inhibition curve obtained by the indirect competitive ELISA with number 6 rabbit antibody.

The inhibition curve was obtained by five replicates. The limit of detection (LOD, also called the least detectable dose) estimated as the concentration of TC giving a 10% inhibition (I_{10} value) of the maximum absorbance, and the working range for ELISA, calculated as the concentration of TC providing a 20−80% inhibition ($I_{20}-I_{80}$ values) of the maximum signal, were 0.01 and 0.1−100 μg/mL, respectively (*26*, *27*). The value of IC_{50} (**Table 2**) was 3.92 μg/mL. The result confirmed that the heterology assay can really improve the sensitivity of TC immunoassays. At the same time, the high sensitivity of the antibody may also be due to the relatively short spacer arm (five atoms) between TC and cBSA. Such a spacer arm can help expose the determinant groups on the immunogen, preventing the determinant groups being masked by the protein tertiary structure (*28*). Moreover, because the hydroxyl group in C6 of TC was used to conjugate protein, this coupling method effectively prevents the formation of lactone and transformation of TC structure. However, the antisera raised against TC-tolidine-BSA and TC-ABA-cBSA have very low inhibition by TC in both homologous assays and heterologous assays. The low chemical stability of TC and the properties (such as length and binging ability) of the spacer should be responsible for the low quality of the antibodies. It is likely that the antibodies produced can recognize only partial structure of TC of the coating antigen and the cavity size of the antibody binding site is not big enough to hold TC. The free TC molecule is, thus, unable to displace the coating antigen from the antibodies (*29*).

Specificity. The specificity of the antibody was evaluated by measuring inhibition curves using three structurally related compounds (TC, chlortetracycline, and oxytetracycline) as competitors and TC-ABA-cOVA as a coating antigen (**Figure 9**). The cross-reactivity studies were carried out by an indirect competitive ELISA by adding various free competitors at different concentrations (ranging from 0.01 to 200 μg/mL) to compete with coating antigen to bind with the antibody to estimate their respective IC_{50} value and then comparing this value with that of TC. The IC_{50} value and cross-reactivity for each compound were given in **Table 2**. These results demonstrated that chlortetracycline showed high cross-reactivity (112%) toward the antibody, whereas oxytetracycline showed a low cross-reactivity (<2%). A chlorine atom at the position of C7 is the only structural difference between TC and chlortetracycline (**Figure 1**). This extra chlorine atom of chlortetracycline does not make any significant change for the affinity of chlortetracycline toward the anti-TC antibody, indicating that the benzene ring in TC system is not an important structural factor to determine the affinity. In the case of oxytetracycline, however, an extra hydroxyl group linked at position of C5 in the molecule makes it demonstrate very low cross-reactivity toward anti-TC antibody, indicating that this hydroxyl group, as a key structural factor, inhibits binding to the TC antibody to result in low affinity. By combining these results, we can conclude that a structural moiety consisting of three nonaromatic rings in TC system is the main antigenic determinant for antibodies developed in this research.

Matrix Effects and Inter- and Intra-assay Variation Determination. It has been known that various substances existing in complex matrixes can affect antigen−antibody interaction in immunoassays. To reduce matrix effects, two

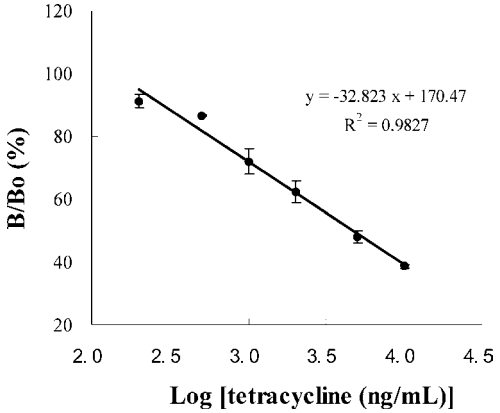

Figure 10. Standard calibration curve. Each point represents the average of five replicates.

Table 3. Inter- and Intra-assay Variation of Raw Milk Spiked with TC

	interassay[a]				intra-assay[b]			
level (ppm)	*n*	measured (ppm)	recovery (%)	CV (%)	*n*	measured (ppm)	recovery (%)	CV (%)
0.2	4	0.16 ± 0.04	80	25.0	5	0.17 ± 0.02	85	11.8
0.5	4	0.46 ± 0.08	92	17.4	5	0.37 ± 0.04	74	10.8
1.0	4	1.00 ± 0.15	100	15.0	5	0.85 ± 0.12	85	14.1
2.0	4	2.32 ± 0.41	116	17.7	5	2.13 ± 0.31	107	14.5

[a] Interassay variation was determined by four replicates on four different days. [b] Intra-assay variation was determined by five replicates on a single day.

common methods could be used. The first one is to clean up the sample, which is time-consuming and laborious and may influence assay reproduction and recovery. The second method is dilution of the extract, which is simple and efficient and is used in this research (*30*). As the dilution of raw milk increased from 1:2 to 1:20, the absorbance gradually increased to approach the PBST buffer values. The average B_0 (antibody binding with no competitor present) from five replicates for milk dilutions at 1:2, 1:10, and 1:20 had absorbencies of 0.869, 0.942, and 1.007, respectively, as compared to 0.997 for antibody in PBST. The IC_{50} values with TC as the competitor were 10.33, 5.50, and 4.06 μg/mL, respectively, as compared with 3.81 μg/mL for PBST. It can be seen that the matrix effect actually can be ignored at a dilution of 1:20, although the difference in IC_{50} values between 1:20 dilution and PBST buffer shows a minor difference indicating a certain level of matrix effect. Thus, a 1:20 dilution was used to perform the inter- and intra-assay variation tests. The milk sample recovery rates were determined from a standard curve (**Figure 10**) with TC concentrations of 0.2, 0.5, 1.0, 2.0, 5.0, and 10 μg/mL in PBST assay buffer. The coefficient of variation for intra-assay was below 15% for intra-assay and below 25% for interassay (**Table 3**). Recovery rates were within 20% of theoretical values, indicating acceptable accuracy.

In summary, the three new conjugates of TC with carrier proteins have been prepared and were used to immunize rabbits to prepare corresponding anti-TC antibodies. An indirect heterologous competitive ELISA has been developed based on an anti-TC antibody prepared from an immunogen of TC-CDI-BSA with TC-ABA-cOVA as the coating antigen. The applicability of the ELISA developed has been used to detect spiked milk samples, and the results have shown that the assay developed could be used as a screening method to detect TC residues in milk.

ABBREVIATIONS USED

MRL, maximum residue level; BSA, bovine serum albumin; OVA, ovalbumin; cBSA, cationized BSA; cOVA, cationized OVA; EDC, 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide; NHS, *N*-hydroxysuccinimide; IC_{50}, concentration at 50% inhibition; Da, unit of molecular mass Dalton; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline Tween 20; OPD, *o*-phenylendiamine; DMF, *N*,*N*-dimethyl formamide; SAS, saturated ammonium sulfate; cFA, complete Freund's adjuvant; iFA, incomplete Freund's adjuvant; ELISA, enzyme-linked immunosorbent assay; CDI, 1,1′-carbonyldiimidazole; TC, tetracycline; tolidine, *o*-tolidine; ABA, 4-aminobenzoic acid.

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