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Anti-oxytetracycline monoclonal antibody based detection of oxytetracycline residue in honey

Shufa Xu [1a], Liwei Hou [1, 2 a], Abebe J. Wubie [1], Tan Cao [1], Yingying Hu [1], Wei Li [1], Zhanbao Guo [1], Jing Zhao [1, 3]* and Jie Wu [1]*

[1] Key Laboratory of Pollinating Insect Biology, Ministry of Agriculture, P. R. China, Institute of Apicultural Research, Chinese Academy of Agricultural Sciences, 100093 Beijing, P. R. China. [2] Food Safety Department, Institute of Agricultural Economics and Development, Chinese Academy of Agricultural Sciences, 100093 Beijing, P. R. China. [3] Bee Products Quality and Supervision Test Center (Beijing), Ministry of Agriculture, P.R.China, Institute of Apicultural Research, Chinese Academy of Agricultural Sciences, 100093 Beijing, P. R. China. e-mail: xushufa@caas.cn, phagraham@yahoo.com, ajenberie@gmail.com

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Abstract

Oxytetracycline (OTC) has the potential to bring severe side effects to humans, when taken from its residues. 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. However, there are no reports concerning the synthesis of OTC immunogen and preparation of anti-OTC antibody use in detection of tetracycline family residues in honey so far. Thus, to identify and understand oxytetracycline pollution in beekeeping industry, we prepared an oxytetracycline immunogen and anti-oxytetracycline monoclonal antibody for the detection of its residues in honey. Conjugates of oxytetracycline (OTC); toluidine BAS and toluidine OVA were prepared as an immunogen and has been used to produce monoclonal antibodies (MAb). A hybridoma 3E3 secreting anti-OTC MAb was obtained and has showed a 50% maximal inhibitory concentration (IC_{50}) value of 0.3 ng ml^{-1} in phosphate buffered saline. With optimum conditions, an indirect competitive ELISA was developed and applied to detect OTC residues in honey samples. As the result, developed ELISA has shown that minimum detection limit of OTC in honey samples was 1.43 ng g^{-1} without cross reactivity to the other tested antibiotics. Optimum CGIA test has also found that minimum detection limit for OTC residues in honey samples to be 2.16 ng g^{-1}. Furthermore, mean recovery percentage of OTC was found to vary from 83.1% to 102.6%. Furthermore, our work has also verified the efficiency of the technique in the detection of the residues in honey. With these all, results have strongly confirmed and recommended that indirect competitive ELISA and CGIA could be used in screening of OTC residues in honey.

Key words: Antibiotics, antibiotic residue, oxytetracycline (OTC), residue detection, monoclonal antibody (MAb), immunogen, enzyme-linked immunosorbent assay (ELISA), colloidal gold-based immunochromatographic assay (CGIA).

Introduction

Tetracycline antibiotics are a group, which is composed of different antibiotics such as tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DC) [1]. The group, in general, is known to inhibit bacterial protein synthesis by blocking attachment of transfer RNA-amino acids to the ribosomes [2-4]. More precisely, they are inhibitors of the codon-anticodon interaction. However, OTC has the potential to bring severe side effects to humans, when taken from its residues. Gastrointestinal, throat sore, liver ache and photosensitive allergic reactions are some of the complications, which could potentially develop from OTC residues in foods.

Furthermore, the abuse of OTC antibiotics in animal husbandry has also confirmed to result in the presence of residues in tissues and even in the environment [5, 6]. In addition, the increased emergence of resistant strains of pathogenic bacteria could have also potential health risks to humans [7, 8]. Nowadays, because of the fact that existing antibiotics are becoming increasingly ineffective in combating microbial infections in humans, antibiotic resistance has also become a global threat [3]. As a result, being consumer protection is a priority; regulatory authorities of different countries have established residue limits for oxytetracycline in edible tissues, milk and honey. Furthermore, they have set the maximum residue limit (MRL) and withdrawal periods. For instance, in the American's food safety cases, the MRLs of OTC in muscle and liver is set to be 2 and 6 ppm, respectively; whereas, 2100 µg kg^{-1} in muscle and 0.05 mg kg^{-1} in honey samples has been set to be the MRLs of OTC in China [4, 9]. Four days of withdrawal period is also set as a requirement for human consumption targeted slaughtered animals in China and European Union (EU) countries. Consequently, residues are being detected from each of the products in various countries.

Recently, development of suitable analytical techniques which are valuable for the detection of oxytetracycline residues, among the tetracycline family, in honey has been considered to be paramount importance and strongly recommended by various scholars [10-13]. Though instrumental analysis methods such as liquid chromatography mass spectrometry (LC-MS) and high-

[a] Shufa Xu and Liwei Hou have the same contribution to this work.
Jing Zhao and Jie Wu are corresponding authors.

performance liquid chromatography (HPLC) are the most widely used methods in the detection of antibiotic residues in food and food products, they have been found to be sensitive, highly specific, requiring extended laboratory facilities and time consuming [10, 14-18].

Therefore, those who are performing routine screens and field detection of OTC have left them because of their less suitability in use. On the contrary, 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 [19].

Though, there are no reports concerning the synthesis of OTC immunogen and preparation of anti-OTC antibody use in detection of tetracycline family residues in honey so far, the ELISA Kits are found to be used from Huaan Maike Co. LTD (Beijing, China) and other research institutes [7, 12].

Thus, the purpose of this work is to disclose the details of OTC immunogen synthesis and characterization of corresponding antibodies. Furthermore, our work has also verified the efficiency of the technique in the detection of the residues in honey.

Materials and Methods

Reagents and animals: Oxytetracycline (OTC), bovine serum albumin (BSA), ovalbumin (OVA), pristane, Freund's complete and incomplete adjuvants, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), carboxymethoxylamine hemihydrochloride (CMO), toluidine, gold chloride trihydrate, MAb isotyping kit, and horseradish peroxidase (HRP)-labelled goat anti-mouse IgG were collected from Sigma-Aldrich (St. Louis, MO, USA). Eight-week-old specific-pathogen-free BALB/c mice were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences, Shanghai, China. The murine myeloma cells Sp2/0 were grown in the basic medium of IMDM medium (Zhongshan Jinqiao, China) supplemented with 0.02 ml ml^{-1} fetal calf serum.

Equipment: During this experiment, model 680 micro plate reader and a BioDot system-dispensing platform were purchased from Bio-Rad (HERCULES, CA, USA) BioDot Inc., Irvine, CA, USA respectively. An SS-4 model automatic guillotine cutter has been collected from Elmwood Park, NJ, USA. Corning Inc. (Cambridge, MA, UK) has supplied (donated) ninety-six-well flat ELISA plates and 96-well flat cell culture plates (catalogue No. 3599).

Buffers: Phosphate-buffered saline (PBS, pH 7.4) composed of 138 mM NaCl, 1.5 mM KH_2PO_4, 7 mM Na_2HPO_4, and 2.7 mM KCl has been used. The wash buffer (PBST) was a PBS buffer containing 0.0005 ml ml^{-1} Tween 20. Sodium borate buffer (pH 8.5) was from 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 mg ml^{-1} OVA and 0.0005 mg ml^{-1} Tween 20.

The substrate buffer was 0.1 M sodium acetate/citrate (pH 5.0). The substrate solution was prepared from 10 mg of OPD dissolved in 25 ml of sodium citrate buffer and then mixed with 5 µl of H_2O_2 [30% (ml ml^{-1})], and 2 M HCl was used as the stopping solution.

Preparation of conjugates of oxytetracycline toluidine BSA and oxytetracycline toluidine OVA: 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 using protocols described by different scholars [4, 20, 21] with certain modifications. Furthermore, since cBSA is better than BSA to couple with functional groups, such as caboxylic groups on hapten[4], BSA was treated with excess ethylenediamine (EDA) for the preparation of cBSA. Then 1 g of BSA (20 µmol) and 100 mg of EDC (300 µmol) mixed in 20 ml of PBS (0.01 M, pH 7.4) was slowly added into a solution of 18 mg of EDA (300 µmol) in 20 ml of PBS (0.01 M, pH 7.4) with continuous stirring to mix them well. The mix was then incubated at room temperature for 2 h and centrifuged at 3000 x g for 5 min. After centrifugation, the mixture was dialyzed with PBS (0.01 M, pH 7.4) to remove free EDA. The solution was also lyophilized and the white solid obtained as a result was stored at 20°C before use in the next steps. Subsequently, the toluidine method was used to prepare immunogen (OTC-toluidine-BSA) and coating antigen (OTC-toluidine-OVA). The immunogen OTC-toluidine-BSA and the coating antigen OTC-toluidine-OVA were prepared using the homobifunctional method (Fig. 1).

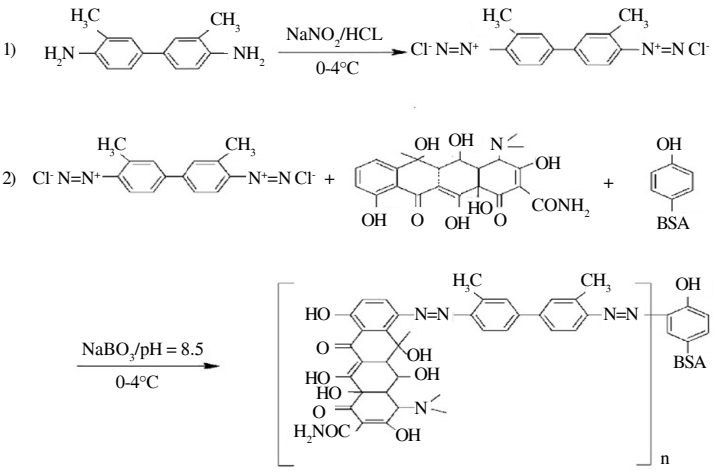

Figure 1. Synthetic procedure for OTC immunogen of OTC-toluidine-BSA through the toluidine method.

Furthermore, 120 µmol of toluidine was dissolved in 5 ml of 0.2 M HCl followed by dropwise addition of 254 µmol of sodium nitrite in 0.5 ml of distilled water at 4°C in the dark with continuous stirring for 30 min [22].

Subsequently, 1.0 ml of orange bis-diazotized toluidine 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 OTC (22.5 µmol). This time, we were able to observe when the color of the mixture solution was changed into purple. Then, the reaction was incubated for 2 h at 4°C in the dark. The purple reaction mixture was dialyzed (mwco, 12,000-14,000 Da) with stirring against PBS (0.01 M, pH 7.4) for three days with frequent changes of the PBS solution to remove the uncoupled free hapten.

The precipitate was removed by centrifugation at 4000 xg and the supernatant was lyophilized to obtain a purple conjugate of OTC-toluidine-BSA and was stored at -20°C for future use [23, 24]. A conjugate of OTC-toluidine-OVA was prepared in a similar method.

Immunization: Eight-week-old BALB/c female mice were vaccinated intraperitoneally with emulsion containing Freund's complete adjuvant and oxytetracycline-toluidine BSA conjugate (1:1; at a dose of 60 µg oxytetracycline-toluidine BSA conjugates

per mouse). Three similar booster vaccines were given in two weeks interval, but the immunogen was emulsified in Freund's incomplete adjuvant. Three days before cell fusion, the mouse was immunized intraperitoneally with 300 µg OTC- toluidine-BSA conjugate without adjuvant.

Preparation of hybridomas: Fused cells were cultured and selected in twenty 96-well cell culture plates with IMDM medium containing hypoxanthine-aminopterin-thymidine (HAT, Sigma-Aldrich) and 0.15 mg ml^{-1} fetal bovine serum (PAA). Eight days after cell fusion, the medium was replaced with IMDM medium containing hypoxanthine-thymidine (HT, Sigma-Aldrich) and 0.15 mg ml^{-1} fetal bovine serum. Again, twelve days, after cell fusion, anti-oxytetracycline antibodies in hybridoma culture supernatants were detected with indirect ELISA and indirect competitive ELISA using oxytetracycline-toluidine-OVA as a coating antigen. Hybridomas, secreting highly specific and sensitive anti-oxytetracycline antibodies, were selected and cloned using limiting dilution method five times to get the hybridoma lines. Stable antibody-secreting clones were expanded and stored in liquid nitrogen.

Production of MAb: The hybridomas were injected intraperitoneally into pristane-primed BALB/c mice to prepare the ascites. After five days, the ascite samples were collected and their titres were detected with indirect ELISA using oxytetracycline-toluidine-OVA conjugate as a coating antigen [9, 25-28]. The class and subclass of the isotypes of the MAb were decided by double immunodiffusion assay with a mouse MAb isotyping kit according to the manufacturer's instruction (Sigma-Aldrich). Anti-oxytetracycline IgG was purified from ascites with an immobilized protein-G affinity column according to the manufacturer's manual (GE Healthcare, Bucks, UK). Purified antibodies were stored at -80°C.

Indirect competitive ELISA and indirect ELISA: Bi-dimensional titration assays were used to determine the most appropriate antibody concentration and the most suitable coating antigen concentration for the competitive ELISA [29-32].

Indirect competitive ELISA was carried out following the standard procedures [31, 33]. Accordingly, the wells of ELISA plate were coated with oxytetracycline-toluidine OVA conjugate at an appropriate concentration in sodium carbonate buffer (2 µg ml^{-1}, pH 9.6) and then coated at 4°C for overnight. The coating wells were blocked with 0.03 mg ml^{-1} dried skimmed milk in phosphate buffered saline (PBS, 300 µl per well, pH 9.7) for 30 min. Then 50 µl of the competitor (standard oxytetracycline or tested antibiotics or samples) and 50 µl of MAb at the most suitable concentration were added into the plates while incubating for 1 h at a temperature of 37°C.

The most appropriate dilution HRP-labelled goat anti-mouse IgG (100 µl per well) was added and incubated for 1 h at a temperature of 37°C. Each of the steps here have been accomplished by washing of the plates with PBS containing 0.005 mL mL^{-1} Tween-20 four times. The HRP tetramethylbenzidine (TMB) substrate solution (100 µl per well) was added and reacted for 15 min at 37°C. The enzyme reaction was terminated by 2 M H_2SO_4 (50 µl per well). The absorbance value at 450 nm was detected with a Bio-Rad model 680 micro-plate reader (Bio-Rad, Hercules, CA, USA).

The indirect competitive ELISA procedure was used also to accomplish the indirect ELISA analysis. According to the indirect ELISA procedure [8, 31, 32], 100 µl of hybridoma culture supernatants or diluted antibodies after blocking were added to the ELISA plate wells with a slight modification.

Cross-reactivity of MAb: Several structurally related or commonly used antibiotics like chlortetracycline, doxycycline, tetracycline, neomycin, gentamycin, kanamycin, sulfamethazine and penicillin G were used for the MAb cross-reactivity analysis with the indirect competitive ELISA using descriptions made before[34]. Accordingly, the cross-reactivity (CR) equation was employed [43] using the following formula:

$$CR\ (\%) = \frac{IC_{50} \text{ of oxytetracycline}}{IC_{50} \text{ of the tested antibiotics}} \times 100$$

Recovery of spiked samples: Here, for this purpose, oxytetracycline-free honey samples were purchased from a local apiary and were identified for the absence of oxytetracycline with HPLC. The honey samples were centrifuged at 5000 rpm for 5 min to remove the fat and precipitate.

To make calibration curves of oxytetracycline in honey, the oxytetracycline stock standard solution (1000 ng ml^{-1}) was prepared. The stock standard solution was diluted with honey to 0, 0.04, 0.12, 0.36, 1.08, 3.24 and 9.72 ng g^{-1} in order to neglect the matrix effects on the immunoassays. The calibration curve for oxytetracycline in honey was then prepared with indirect competitive ELISA. For the recovery assay, oxytetracycline-spiked solutions were prepared by dissolving oxytetracycline in honey to obtain final concentrations of 1.0, 5.0, 10.0 and 20.0 ng mmol l^{-1} with PBS. The recovery of oxytetracycline from the spiked honey was obtained from the calibration curve, prepared by the indirect competitive ELISA[4, 35].

Colloidal gold-based immunochromatographic assay (CGIA):
Colloidal gold-labelled MAb: Fifty-nm-size colloidal gold was prepared [36]. The colloidal gold-labelled MAb was also prepared as described by Verheijen *et al.* [21]. Furthermore, the optimum MAb concentration for labeling was determined as described by different researchers [28, 31, 32]. 1000 µg of Purified MAb in 1 ml Milli-Q purified water was slowly added to 100 ml of colloidal gold solution (pH 8.0) and was stirred continuously for 20 min at room temperature. Then 10 ml of 0.05 mg ml^{-1} BSA solution was added into the mixture and was stirred for another 20 min at room temperature; thereafter, it was centrifuged with 10,000 xg and 4°C for 30 min.

After centrifugation, the precipitate of the gold-labeled MAb was re-suspended with 0.01 M PBS (pH 7.4) containing 0.05 mg ml^{-1} polyethylene glycol (PEG)-1500 and 0.01 mg ml^{-1} sodium azide and was centrifuged again. The precipitate was then re-suspended with 5 ml of 10 M PBS (pH 7.4) containing 0.02 mg ml^{-1} BSA and 0.01 mg ml^{-1} sodium azide and stored at 4°C for further use.

CGIA development: CGIA was developed using methods described before [8]. Briefly, the sample absorbent and the conjugate pads were treated with PBS (20 M, pH 7.4) containing 0.02 mg ml^{-1} BSA, 0.02 mg ml^{-1} sucrose and 0.001 mg ml^{-1} sodium azide, and were dried for 3 h with a temperature of 37°C. The sensitivity, specificity and incubation time of CGIA were used to determine the optimal

immobilization concentrations of oxytetracycline-BSA conjugate, gold-antibody conjugate and goat anti-mouse antibodies. At optimal conditions, oxytetracycline-BSA (0.21 mg ml^{-1}) and goat anti-mouse antibodies (1 mg ml^{-1}) were dispensed into the nitrocellulose membrane of the test and control lines with a Quanti 3000 BioJets attached to a BioDot XYZ-3000 dispensing platform and was dried for 3 h with a temperature of 37°C. Furthermore, gold-labelled MAb was dispensed into treated conjugated pad at a jetting rate of 7 µl cm^{-1} and was dried. The treated nitrocellulose membrane, the prepared conjugated pad, the sample pad and the absorbent pad were assembled as immunochromatographic strip. The assembled plate was cut into strips (60 mm × 4 mm) with an AZCON Sur-Size automatic guillotine cutter.

CGIA procedure: Sample drops (about 100 µl, pH 7.0) were added into the sample absorbent pad, as a result, the sample was migrated into the conjugate pad, and the colloid gold-labelled MAb on the conjugate pad was solublized and began to migrate along with the sample up to the nitrocellulose membrane. The colloid gold-labelled MAb was captured by the oxytetracycline-toluidine BSA conjugate immobilized on the membrane to display a clear red test line. The excess colloid gold-labeled MAb migrated farther was captured by the goat anti-mouse antibodies to display a red control line.

Data analysis: According to the absorbance and the logarithm of analyte concentration from these steps, the standard curves were established. Data analysis, in this case, was done using Microsoft® Excel 2007. The 50% maximal inhibitory concentration (IC_{50}) values were also calculated based on sigmoidal curves, which were obtained based on a four-parameter logistic equation [25, 31, 32].

Results

Preparation of oxytetracycline-toluidine BSA and oxytetracycline-toluidine OVA conjugates: As a small molecule with the molecular weight of 461.4, OTC is not able to elicit the immune response of animals to produce antibodies. To make it immunogenic, it has been elaborated that it must be conjugated with a carrier protein before immunization. Among protein carriers, BSA and OVA are the two most fitful ones.

Proper follow up of the procedures resulted that preparation of antigens and ultraviolet scan analyses confirmed that scan curves of BSA, OTC, toluidine, and OTC-toluidine-BSA conjugates have shown significant difference (Fig. 2 http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2801090/figure/F1/). This result has also revealed that oxytetracycline has successfully conjugated with both carrier proteins, BSA and OVA.

On the other hand, the result has indicated that BSA has absorbance peaks of 246 and 278 nm, which might have come from aromatic groups in the molecule. In addition, toluidine also has shown to have two peaks at 228 and 281 nm with a red shift as compared with BSA's 278 nm. This might be because toluidine has a longer conjugate system consisting of two benzene rings. In the OTC-toluidine-BSA conjugate, conjugation system has been expended significantly, because tolidine, which has been found to consist two benzene rings, links two aromatic systems together to form the conjugate. The two nitrogen double bonds, working as two bridges in OTC-toluidine-BSA, connect two small conjugation systems together to form a larger one (Fig. 2). Consequently, we found a peak at a long wave length of 385 nm in UV spectrum for OTC-toluidine-BSA as a result of red shift in longer conjugation system as it was expected. The corresponding coating antigen OTC-toluidine-OVA has been confirmed also to give a similar pattern in UV spectrometry.

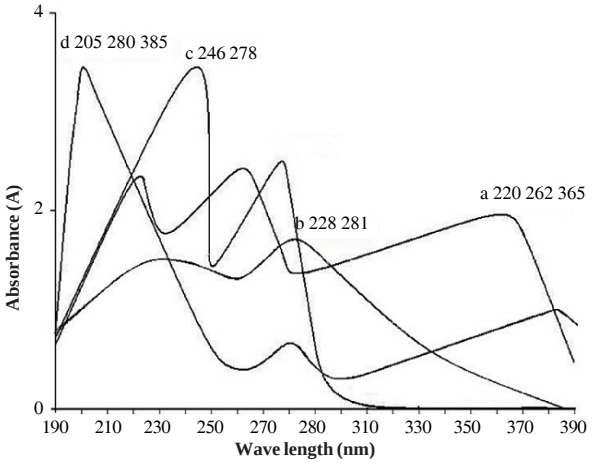

Figure 2. UV absorbance spectrum of oxytetracycline, toluidine, BSA and oxytetracycline-toluidine-BSA.
(a) OTC, (b) toluidine (c) BSA,(d)OTC-toluidine-BSA.

Hybridoma production and monoclonal antibody characterization: Oxytetracycline-toluidine-BSA conjugate was used as an immunogen for immunization of the experimental mice, while oxytetracycline-tolidine-OVA conjugate was coated onto ELISA plates to determine the titer and inhibition level of antibodies. From this procedure, we have confirmed that four out of eight mice immunized with oxytetracycline-toluidine-BSA have produced high titer (1:100,000) antisera against oxytetracycline and were chosen for further hybridoma production. The results of titer detection are indicated in Table 1.

Mice number four (out of the total immunized and produced high titer (1:100,000) antisera against oxytetracycline), was randomly selected to face cell fusion experiment. Consequently, from this experimentation, hybridomas were obtained from the initial culture plate wells. As a result of screening by an indirect ELISA, nine hybridoma culture plate wells consisting of 1E12, 1G2, 2B1, 2H1, 2H7, 3E3, 3E8, 5A7 and 5E11 were found with positive reactions. Determination of hybridoma's sensitivity to recognize un-conjugated oxytetracycline-toluidine-BSA and oxytetracycline-toluidine-OVA was further assayed with an indirect competitive ELISA (Table 2).

Table 1. Detection results of titer antisera against oxytetracycline.

Mouse	Dilution times of antisera											
no.	200	400	800	1600	3200	6400	12800	25600	51200	102400	blank	negative
#1	0.951	1.030	1.097	0.980	0.985	1.075	0.954	0.839	0.678	0.509	0.019	0.117
#2	1.063	1.071	1.118	1.166	1.122	1.029	0.917	0.836	0.788	0.590	0.016	0.118
#3	0.913	0.985	1.020	1.024	1.024	1.061	0.957	0.855	0.700	0.576	0.018	0.120
#4	1.014	1.044	1.058	1.087	1.100	1.032	0.982	0.868	0.615	0.458	0.016	0.101

Each point represents the average of five replicates.

Table 2. Indirect competitive ELISA for determining sensitivity of hybridomas.

Cell No.	BSA screening	OVA screening
1E12	0.017	0.016
1G2	0.018	0.018
2B1	0.103	0.024
2H1	0.061	0.011
2H7	0.242	0.023
3E3	0.943	0.945
3E8	0.016	0.014
5A7	0.033	0.016
5E11	0.158	0.004
negative	0.014	0.006
blank	0.012	0.003
positive	0.886	1.001

Each point represents the average of five replicates.

This sensitivity detection assay revealed that three hybridomas (2H7, 3E3 and 5E11) were found with the highest ability to secrete anti-oxytetracycline antibody. The relative OD values and the curves from this sensitivity assay has been described in Table 3 and Fig. 3.

Table 3. Indirect competitive ELISA to determine antibody competitive sensitivity with OTC.

OTC concentration ng mL^{-1}	9.72	3.24	1.08	0.36	0.12	0.04	0
2H7 (1:100)	0.70	0.51	0.40	0.32	0.18	0.15	0.10
3E3 (1:100)	0.89	0.80	0.74	0.59	0.42	0.38	0.33
5E11 (1:50)	0.26	0.28	0.38	0.34	0.38	0.38	0.31

Each point represents the average of five replicates.

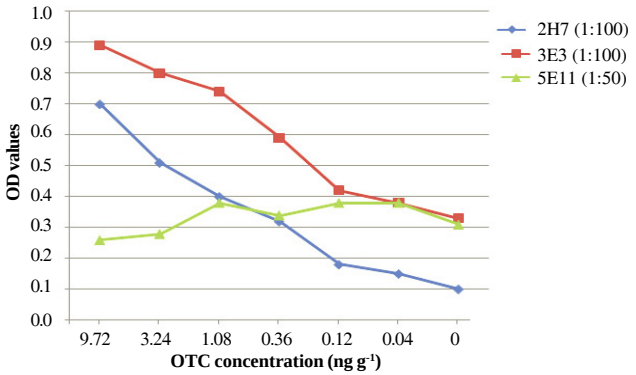

Figure 3. Indirect competitive ELISA for determining antibody competitive sensitivity with OTC.
Each point represents the average of five replicates.

The results obtained from the sensitivity detection assay showed that only culture plate wells 2H7 and 3E3 were found to be sensitive to OTC molecule. However, culture plate well 5E11 was not found to be competitive to OTC. This might be due to its degeneration for uncertain reasons during experimentation. The other most important result obtained has confirmed that with 4 ng ml^{-1} concentration of OTC inhibition rate of MAb 3E3 and MAb 2H7 was found to be 28.4 and 19.6%, respectively. This, therefore, showed that these two monoclonal antibodies could be successfully employed in further detection of OTC residues in honey.

However, IC_{50} value of MAb 3E3 in PBS buffer was 0.3 ng ml^{-1}. This has also confirmed that MAb 3E3 is much more sensitive than that of Mab 2H7. Thus, this MAb has been selected for further production of monoclonal antibodies and this mouse MAb isotyping kit, 3E3, was determined to be the subclass IgG_{2b} with a kappa light chain (Table 4).

Table 4. Subclass determination of the prepared antibody.

Iso-typing	3E3	NC
M	0.094	0.066
G1	0.100	0.087
G2a	0.080	0.067
G2b	0.223	0.095
G3	0.103	0.080
A	0.072	0.072
K	0.195	0.080
λ	0.074	0.077

Used a mouse MAb isotyping kit, 3E3 was determined to be the subclass IgG_{2b} with a kappa light chain. Each point represents the average of five replicates.

Titres of hybridoma 3E3 supernatant and ascitic fluid determined by indirect ELISA are found to be 1:5,000 and 1:500,000, respectively. The IgG yield of purified MAb was 5.72 mg ml^{-1}.

Cross-reactivity of MAb: As a result of MAb specificity evaluation with the indirect competitive ELISA using different antibiotics as competitors, Mab 3E3 has negligible cross-reactivity (<0.01) with other antibiotics (neomycin, gentamycin, kanamycin, sulfamethazine and penicillin G) and the tetracycline family (tetracycline, chlortetracycline and doxycycline) (Table 5).

Table 5. Cross-reactivity of the anti-oxytetracycline MAb with different antibiotics.

Antibiotics	IC_{50} (ng mL^{-1})	Cross-reactivity (CR %)
Oxytetracycline	3.0	100
chlortetracycline	>50 000	<0.01
doxycycline	>50 000	<0.01
Tetracycline	>50 000	<0.01
Neomycin	>50 000	<0.01
Sulfamethazine	>50 000	<0.01
Kanamycin	>50 000	<0.01
Gentamycin	>50 000	<0.01
Penicillin G	>50 000	<0.01

The cross-reactivity (CR) was calculated using the equation: CR (%) = **(**IC_{50} of oxytetracycline**) (**IC_{50} of the tested antibiotics**)**$^{-1}$×100 (1). Each point represents the average of five replicates.

Indirect competitive ELISA kit: From bi-dimensional titration assays, optimum levels of indirect competitive ELISA for optimum concentrations of coating antigen, MAb and HRP-labelled goat anti-mouse IgG for honey samples were 2.295, 0.014 and 0.001 µg g^{-1}, respectively. The optimum incubation period for the assays were found to be overnight at 4°C for antigen coating, 1 h at 37°C for MAb (with samples and HRP-labelled goat anti-mouse IgG) and 15 min at 37°C for substrate color development.

Under optimum conditions, the regression equation for Logit Log calibration curve was expressed as y = -0.826x + 2.318 (with correlation coefficient, r = 0.993) and the calibration curves were obtained. Thus, the result has shown that the range of oxytetracycline calibration curve (for IC_{88} to IC_5) was found to be in between 0.04 and 2.86 ng g^{-1} in the collected honey samples (Fig. 4) and the minimum oxytetracyline detection limit was found to be 1.43 ng g^{-1}.

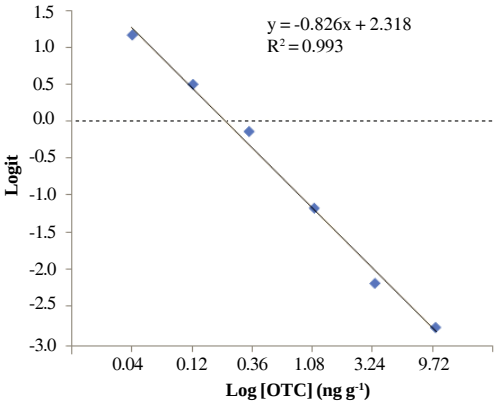

Figure 4. Calibration curves of monoclonal antibody from 3E3.
Logit = ln (p q^{-1}), p=B B_0^{-1}, q = 1-p, B = OD value of positive, B_0 OD value of 0 µg kg^{-1}. The regression equation of Logit/Log calibration curve was expressed as follows: y= -0.826x+2.108 (correlation coefficient, r = 0.993). Each point represents the average of five replicates.

Analysis of spiked samples with ELISA: Based on the indirect competitive ELISA analysis of honey samples spiked with various amounts of oxytetracycline (1.0, 5.0, 10.0 and 20.0 ng g^{-1}) to validate the developed ELISA, calibration curve values indicated that recoveries of oxytetracycline were ranged from 83.1 to 102.6% (Table 6). This, furthermore, indicated that recoveries of ELISA are sufficient to analyze oxytetracycline residues in honey samples.

Table 6. Recoveries of oxytetracycline from spikedhoney (n=10).

Level added (ng g^{-1})	Level found (ng g^{-1})	Recovery rate (%)
0	0	-
1.0	8.5±2.6	85.0
5.0	51.3±2.0	102.6
10.0	98.5±1.1	98.5
20.0	166.2±1.9	83.1

Recovery rate (%) was expressed as: Recovery rate (%)=(Cx-C_0) C^{-1}×100, where Cx is the calculated concentration based on the calibration curve equation as the samples spiked with oxytetracycline, C_0 is the calculated concentration based on the calibration curve equation as the samples concentration is 0 µg kg^{-1}, and C is the concentration of spiked oxytetracycline in the experiment.

CGIA: The CGIA conditions were optimized for high sensitivity without any nonspecific bindings. Oxytetracycline-free honey spiked with oxytetracycline with different levels (0, 0.36, 0.72, 1.08, 1.44, 2.16, 2.88 and 3.6 ng g^{-1}) were detected with the CGIA and estimated for the reliability of the assay.

From this result, it has been confirmed that red color of the test line on the strip has gradually thinned with an increasing amount of oxytetracycline and completely disappeared at 2.88 ng g^{-1} of oxytetracycline in the honey samples (Fig. 5). The lower detection limit of the oxytetracycline assay has been therefore defined as least concentration of oxytetracycline in the sample at which the color of the test line is weaker than that of the control line.

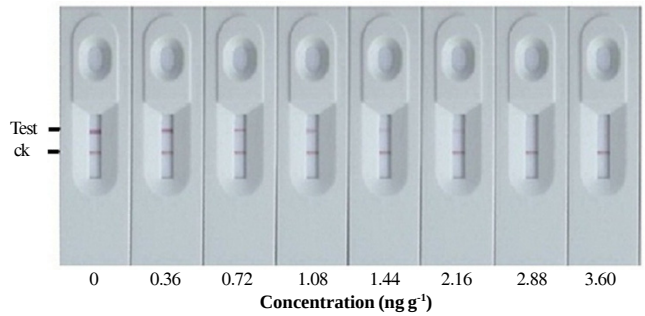

Figure 5. Detection of OTC with the CGIA.

As shown in Fig. 5, 2.16 ng g^{-1} of oxytetracycline caused slight but distinguishable degressive color intensity with naked eyes compared with the control line. Thus, the presence of 2.16 ng g^{-1} of oxytetracycline in PBS have been confirmed as the lowest detection limit of CGIA in honey samples, which is far below than the EU maximum residue limit (MRL) (50 ng mg^{-1}).

From the analysis made to understand and determine the specificity of the CGIA, 1000 ng ml^{-1} of neomycin, gentamycin, kanamycin, sulfamethazine, penicillin G, chlortetracycline, doxycycline, tetracycline and oxytetracycline in PBS were detected (Fig. 6) and their result were negative. This indicates that the assay is specific to oxytetracycline and even to its family (with very similar structures). Therefore, it has been indicated that the developed CGIA is suitable for the screening of oxytetracycline residues in honey samples.

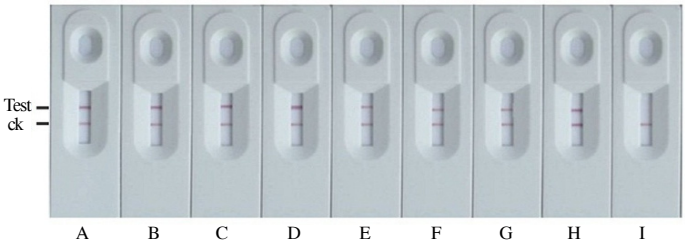

Figure 6. Specificity of the CGIA.
A: neomycin; B: gentamycin; C: kanamycin; D: sulfamethazine; E: penicillin G; F: chlortetracycline; G: doxycycline; H: tetracycline; I: oxytetracycline.

Applications of CGIA and indirect competitive ELISA in honey samples: CGIA detection for oxytetracycline residue from 25 farm gate honey samples revealed that only two samples were found to contain the residue. Further confirmation of positive samples using indirect competitive ELISA oxytetracycline calibration curve has elaborated that concentrations of the two positive samples were found to be 2.70 and 3.20 ng g^{-1}, respectively.

Discussion

In this study, MAb 3E3 specific for the structure of oxytetracycline was successfully prepared. This also has shown no cross-reactivity with the other tested antibiotics, such as neomycin, gentamycin, kanamycin, sulfamethazine, penicillin G and even with the tetracycline family which are very similar in structure [2]. Using the MAb, a highly sensitive and specific ELISA and CGIA for detection of oxytetracycline residues in honey samples have been successfully developed. The analysis of spiked samples proved that the two immunoassays are suitable to detect oxytetracycline residues in honey samples. Seldom, data had shown that no cross-reactivity was observed with the structurally similar compounds[37]. Compared to other methods, the immune-method was superior in terms of detection limit, dynamic range, and recovery with simple sample preparation.

It has been clarified that as a small molecule, OTC is not able to elicit the immune response of an animal to produce the anti-OTC antibody and is, therefore, non-immunogenic. Conjugation to a carrier protein before immunization is also known to produce immunogenic anti-OTC antibody. Among protein carriers, BSA and OVA are the two most commonly used ones, and usually they have been proved to give satisfying results. In this experiment, BSA was treated with an excess of ethylenediamine (EDA) as

described by different scholars to convert carboxylic acid groups into primary amine groups to prepare cBSA [4, 22, 37].

The cBSA prepared has been elaborated for its 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 [37-39].

With the appropriate manipulation of the procedures in this research, OTC-toluidine-BSA was synthesized using toluidine, a homobifunctional cross-linking agent, as a bridge to link OTC and carrier protein BSA by a simple one-step conjugation reaction.

The two amino groups in toluidine were diazotized to create the requisite bis-diazonium derivative and then coupled simultaneously the para position of the aromatic hydroxyl group of OTC with the ortho position of tyrosine residue in BSA, forming azo derivative. The diazo reaction proceeded by electrophilic attack of the diazonium group towards the electron-rich points on the target molecules. 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 [4]. Both the para and the ortho positions of the aromatic hydroxyl group of OTC have no substituent; thus, the diazo reaction is more likely to proceed at the para position of OTC according to the reason mentioned above. For tyrosine side chains of BSA, only the ortho modification is possible.

Recently, three of antibiotic residue detection methods are used (the microbiological assay, instrument method and immunoassay method). Microbiological assay method, because of its convenience, low cost and broad spectrum characteristics [40, 41], is used in antibiotics screening in foods. However, this method in the laboratory normally is slow and with low sensitivity [21].

The instrument methods used to analyze oxytetracycline are gas chromatography, high performance liquid chromatography (HPLC) and liquid chromatography with mass spectrometric detection (LC-MS) [13, 15, 17, 18]. Though these methods are sensitive and highly specific, they require expensive instrumentation and highly skilled manpower. Furthermore, they are blamed to be time-consuming, expensive and are not suitable for routine analysis of large-scale samples.

On the other hand, immunoassay has been used as an alternative to the instrument and microbiological methods for accurate measurements of antibiotic residues in complex matrices. This is because; it is highly sensitive and specific, easy to manage during large-scale analysis, requires low cost and is known to be rapid in its results.

Unlike the instrument methods, immunoassays do not require sample pre-concentration and extraction. Therefore, they could be used extensively in detecting trace amounts of chemicals [9, 16, 19, 20, 26, 33, 35, 41, 43].

In addition, use of immunoassays in detection of antibiotic residues in animal samples based on polyclonal antibodies and monoclonal antibodies (MAb) have been well described by different scholars [7, 19, 23, 24, 34, 40, 42].

In this paper, an indirect competitive enzyme-linked immunosorbent assay (ELISA) and colloidal gold-based immunochromatographic assay (CGIA) using an anti-oxytetracycline MAb were developed and applied to detect oxytetracycline residues in honey samples. The product, if oxytetracycline exists in the sample, will compete with the immobilized conjugate on the test line to bind the limited amount of colloid gold-labelled MAb.

As a benchmark, the more oxytetracycline that exists in the sample, the weaker color will be observed at the test line. The other observation was color intensity decreases with increasing oxytetracycline concentration in the sample. When oxytetracycline exists in a sufficient amount, it will completely prevent the binding of the colloid gold-labelled MAb with the immobilized OTC-toluidine BSA conjugate on the test line and there will not be visible test line except one red control line. In addition, if the control line has no color, the assay shall be considered as invalid. Moreover, it has been proved that raw honey samples need not to receive pretreatment for CGIA, developed in this study.

Since all required reagents are included in the strip, the described CGIA is found to be easy to perform and its result can be obtained within 3-5 min without any equipment. Hence, the result from CGIA is visible and can be used as a convenient qualitative and/or semi-quantitative method of rapid screening of honey samples for oxytetracycline residues above 3.0 ng ml^{-1} of the detection limit, which is far lower than EU MRL level (50 ng mg^{-1}). Therefore, the assay is confirmed especially to be suitable for on-site screening of honey samples.

The results of the experimental procedures have been notably confirming that two of the assays can be eventually used for efficient and accurate detection of oxytetracycline residues in other animal food products such as milk, fishes, chicken and eggs if fruitful treatment has been conducted prior to detection.

Conclusions

The results of spiked analysis and specific analysis have demonstrated that indirect competitive ELISA and CGIA could be applied for the screening of OTC residues in honey samples.

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