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# Development of Oxytetracycline Test Kit Using Enzyme-linked Immunosorbent Assay Technique

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**Abstract.** In Thailand, farmers often use oxytetracyline (OTC) to protect and treat shrimps from disease. The widespread use of OTC could lead to OTC residues in food-producing animal. To prevent consumers from exposure to drug residues and increasing in drug resistance pathogens, several regulatory authorities around the world have set the maximum residue limits (MRLs) for OTC and the surveillance detection programs are enforced. Therefore, an effective screening method is essential to detect OTC in food. Enzyme-linked immunosorbent assay (ELISA) is a suitable method for screening a large number of samples due to its simplicity, rapidity, cost effectiveness, high sensitivity and high specificity. The aim of this study was to evaluate the effectiveness of the antibody (clone 2-4F) in different ELISA formats, including antibody-captured competitive indirect ELISA, antibody-captured competitive direct ELISA and antigen-captured competitive direct ELISA, in order to detect OTC. The results showed that the antibody-captured competitive direct format was the most suitable one with the 50% inhibition concentration ($IC_{50}$) and the limit of detection (LOD) of 11.5 ng/ml and 1.55 ng/ml, respectively. The detection range of the prototype test kit was between 0.4 ng/ml and 128 ng/ml which covered the current MRLs. In addition, the specificity of this prototype was also tested. The antibody cross-reacted with other tested antibiotics in tetracycline group but did not cross-react wit other unrelated compounds. Taken together, we concluded that the monoclonal antibody from hybridoma clone 2-4F can be used in the antibody-captured competitive direct ELISA format to detect OTC with an acceptable sensitivity and specificity level.

**Keywords:** oxytetracycline, ELISA, test kit, monoclonal antibody

## 1. Introduction

Tetracyclines (TCs) are a group of broad spectrum antimicrobial and bacteriostatic drug which consist of tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC). They are actively transported into the cells of susceptible bacteria and inhibit protein synthesis [1].

TCs have been used routinely for veterinary and human medicine in prevention and treatment of infectious diseases. In Thailand, OTC is used to prevent bacterial infection in shrimp farming. It is usually administered through medicated feed during the growing period with a recommended withdrawal period of 4-16 days before harvesting, depending on the OTC concentration and shrimp species [2,3]. Improper use of TCs has an impact on the human health in two main directions. At high concentration, TCs inhibit mammalian protein synthesis, while at low concentration, they cause acquired resistant to microorganisms [4]. In order to prevent harmful health effects to consumers due to TCs residues, the maximum residual limits (MRLs) for animals for consumption have been established. The European Union (EU) proposed MRLs of 100 ng/g for muscle, 300 ng/g for liver and 600 ng/g for kidney for all animals for human

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consumption [5]. These limits require the development of sensitive and specific methods for the detection of OTC in food-producing animal. Until now, various methods have been reported, most of which are base on chemical methods such as high performance liquid chromatography (HPLC) [6-9], liquid chromatography-mass spectroscopy (LC-MS) [10] and capillary electrophoresis [11]. Although these methods have high precision and accuracy, they require expensive equipments and trained professionals.

In contrast, Enzyme-linked immunosorbent assay (ELISA), an immunological method, become the popular method for routine screening and field detection because of its high sensitivity, simplicity, cost effectiveness and ability to screen large number of sample. In Thailand, the OTC ELISA test kit is in high demand for residue determination in food. However, this commercially available test kits must be imported from abroad. Therefore, the objective of this study was to develop a domestic ELISA test kit for OTC detection.

# 2. Methods

## 2.1. Antibody production and purification

Hybridoma clone 2-4F ($IgG_1$) specific for OTC from the Institute of Biotechnology and Genetic Engineering, Thailand (IBGE) [12] was cultured in 500 ml spinner flask containing serum free medium and incubated in 5% $CO_2$ at 37ºC. Hybridoma culture media was collected and detected the antibody by indirect competitive ELISA.

Anti-OTC monoclonal antibody (Anti-OTC-MAb) was purified from hybridoma culture media by affinity chromatography method using Protein G sepharose column. Antibody in culture media was applied to Protein G in the column. After elution of unbound substances with 0.1 M phosphate buffer pH 8.0, MAb was eluted from the column with 0.1 M citrate buffer pH 3.0 with a flow rate of 1 ml/min. Fractions were collected at 1 ml/tube and adjusted pH to 7.0 with 1.0 M Tris-HCl buffer pH 9.0. Each fraction was measured absorbance at 280 nm. Fractions with the value higher than 0.3 were pooled and dialyzed against 0.01 M phosphate buffer saline pH 7.4 (PBS) for 5 times. Protein concentration was determined using BCA protein kit (Pierce).

Hybridoma culture supernatants and purified MAb were checked for purity and molecular weight by sodium dodecyl sultfate polyacrylamide gel electrophoresis (SDS-PAGE), using 10% separating gel and 5% stracking gel.

## 2.2. Conjugation of OTC to OVA and HRP

OTC was conjugated to a carrier protein (ovalbumin; OVA) or labelled enzyme (horseradish peroxidise; HRP) using Mannich reaction [13]. Four mg of OTC were dissolved in 0.2 ml 30% ethanol and mixed with protein or enzyme (10 mg OVA or HRP in 0.3 ml distil water). Three molar of sodium acetate buffer pH 5.5 (0.2 ml) and 7.5% (v/v) formaldehyde (0.2 ml) were added and stirred overnight at room temperature. The mixture was dialyzed against PBS for 5 times and kept at -20ºC until use.

## 2.3. Conjugation of Anti-OTC-MAb to biotin

Anti-OTC-MAb was dialyzed in 0.1 M carbonate buffer pH 8.0 for 3 times. Aminohexanoyl-Biotin N-Hydrozysuccinimide (AH-BNHS) was added into anti-OTC-MAb solution with a ratio of 1:10 (w/w) and kept at room temperature for 4 hr. The MAb-biotin conjugate was dialyzed against PBS for 5 times and kept at -20ºC until use.

## 2.4. ELISA test kit development

### 2.4.1 Antibody-captured competitive indirect ELISA (Goat anti-mouse IgG-HRP)

A 96-well plate was coated with 5 µg/ml OTC-OVA in PBS 100 µl/well by incubation at 4ºC overnight. The plate was washed three times with PBS containing 0.05% Tween-20 (PBST) and blocked with 5% (w/v) skimmed milk powder in PBS (300 µl/well) by incubation at 37ºC for 1 hr. The plate was washed and incubated at 37ºC with a mixture of 0.03 µg/ml anti-OTC-MAb and various concentrations of OTC between 0.488 and 2,000 ng/ml for 2 hr. The plate was washed again and further incubated with goat anti-mouse IgG-HRP (1:10000 in PBS, 100 µl/well) at 37ºC for 1 hr. Substrate solution was prepared from 250 ml of 41 mM

3, 3', 5, 5'-Tetramethylbenzidine (TMB) in DMA dissolving in 10 ml of 205 mM potassium citrate buffer pH 4.0 with 3.075 mM $H_2O_2$ [14]. After another washing, substrate solution was added 100 µl/well and incubated at room temperature in the dark. After 15 min, the reaction was stopped with 100 µl/well of 1 M $H_2SO_4$. The absorbance at 450 nm was read using a microplate reader.

### 2.4.2 Antibody-captured competitive direct ELISA (Streptavidin-HRP)

A 96-well plate was coated and blocked as same as described in 2.4.1. The plate was washed and incubated at 37ºC with a mixture of 0.06 µg/ml MAb-biotin and various concentrations of OTC between 0.488 and 2,000 ng/ml for 2 hr. The plate was washed again and further incubated with Streptavidin-HRP (1:4000 in PBS, 100 µl/well) at 37ºC for 30 min. After another wash, 100 µl/well of substrate solution was added and incubated at room temperature in the dark. After 15 min, the reaction was stopped with 100 µl/well of 1 M $H_2SO_4$. The absorbance at 450 nm was read using a microplate reader.

### 2.4.3 Antigen-captured competitive direct ELISA

A 96-well plate was with 1.25 µg/ml anti-OTC-MAb and blocked as same as described in 2.4.1. The plate was washed and incubated at 37ºC with a mixture of 1.5 µg/ml OTC-HRP and various concentrations of OTC between 0.488 and 2,000 ng/ml for 2 hr. After another wash, 100 µl/well of substrate solution was added and incubated at room temperature in the dark. After 15 min, the reaction was stopped with 100 µl/well of 1 M $H_2SO_4$. The absorbance at 450 nm was read using a microplate reader.

## 2.5. Evaluation of the test kit

The sensitivities in terms of limits of detection (LOD) and inhibition concentration at 50% ($IC_{50}$) of each ELISA types were studied. The LOD was calculated by subtracting the concentration at $B_0$ with three times of its standard deviation (SD) obtained from the direct and indirect competitive ELISA without competitor of 9 replicates.

The $IC_{50}$ was calculated and defined as the concentration at which 50% $B/B_0$ was obtained, where B and $B_0$ are the average of absorbance obtained from the direct and indirect competitive ELISA with and without different concentrations of OTC, respectively.

The specificity was evaluated in term of the cross reactivity with other antibiotics. $IC_{50}$ of interested compounds were obtained in the same manner previously described. Then the degree of the cross-reactivity was calculated using the following formula:

$$\%\ \text{cross reactivity} = \frac{IC_{50}\ \text{of OTC}}{IC_{50}\ \text{of competitor}} \times 100$$

## 3. Result and discussion

Anti-OTC-MAb from hybridoma (2-4F) culture media was purified by Protein G sepharose affinity column. Eluted fraction No. 4 to 15 showed absorbance at 280 nm higher than 0.3, indicating high protein contents. Therefore, these fractions were pooled and dialyzed.

After purification, the molecular weight and purity of anti-OTC-MAb were checked by SDS-PAGE. As shown in Figure 1, the heavy chain and light chain of antibody at 59 kDa and 26 kDa, respectively, were evident, which are in agreement with values previously reported [15]. However, the antibody was not completely purified because there were two extrabands above the heavy and light chains at 65 kDa and 29 kDa. Nevertheless, the partially purified antibody was pure enough for using in further investigation.

Each format of ELISA was optimized for the concentration of antigen and antibody. In both Ab-captured competitive formats, the plates were coated with antigen (OTC-OVA) at 5 µg/ml and MAb and MAb-biotin was used at 0.03 and 0.06 µg/ml, respectively. In Ag-captured competitive direct format, the plate was coated with optimized concentration of antibody 1.25 µg/ml and antigen at 1.5 µg/ml. The $IC_{50}$ and LOD of all formats were analyzed from the curve of the relationship between OTC concentrations and $\%B/B_0$ (figure 2) and in table 1. All formats were sensitive enough to detect OTC at the concentrations lower than the MRL level; however, Ab-captured competitive direct ELISA was the most sensitive format with the $IC_{50}$ of 11.5 ng/ml and LOD of 1.55 ng/ml. Therefore, this format was chosen for development of OTC ELISA test kit prototype with the detection range of standard curve between 0.4 and 128 ng/ml (figure 3).

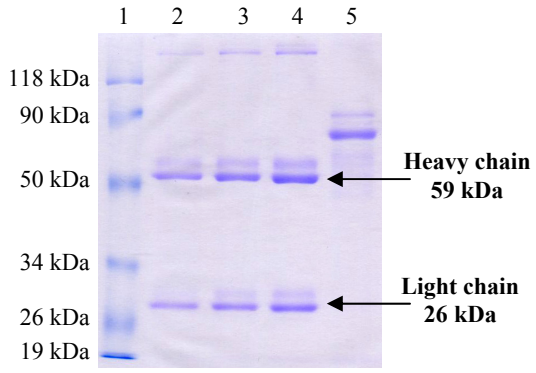


Figure 1. SDS-PAGE of antibody: lane 1, standard protein marker; land 2, 3 and 4, purified antibody at 2, 3 and 5 µg, respectively and lane 5, serum free culture media at 5 µg.

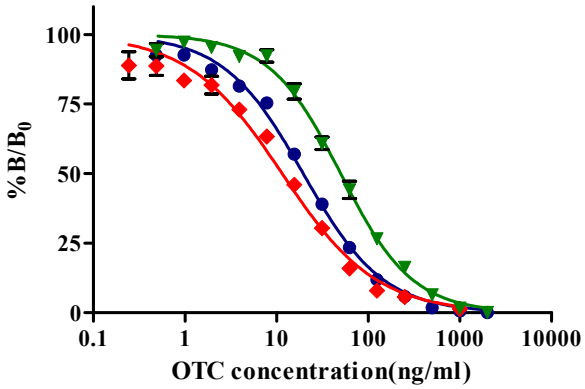


Figure 2. The inhibition curve of each ELISA format: Ab-captured competitive indirect (—●—), Ab-captured competitive direct (—◆—) and Ag-captured competitive direct (—▼—).

Furthermore, the cross-reactivity of the test kit, which is one of the major criteria for test kit development, was also considered in the test development. The cross-reactivities of the test kit to some selected antibiotics within the TCs group and other unrelated groups were shown in table 2. The results showed that the antibody cross-reacted to RTC at 387% and to other antibiotics in TCs group in the range of 0.16-6.6%. Importantly, it did not cross-react to other tested compounds (< 0.01%). This result indicated that the antibody is suitable for test kit development to detect OTC and RTC.

Table 1. Sensitivity of MAb in different ELISA formats

| Format of ELISA | $IC_{50}$ (ng/ml) | LOD (ng/ml) |
|---|---|---|
| Ab-captured competitive indirect | 19.5 | 1.75 |
| Ab-captured competitive direct | 11.5 | 1.55 |
| Ag-captured competitive direct | 50.1 | 5.16 |

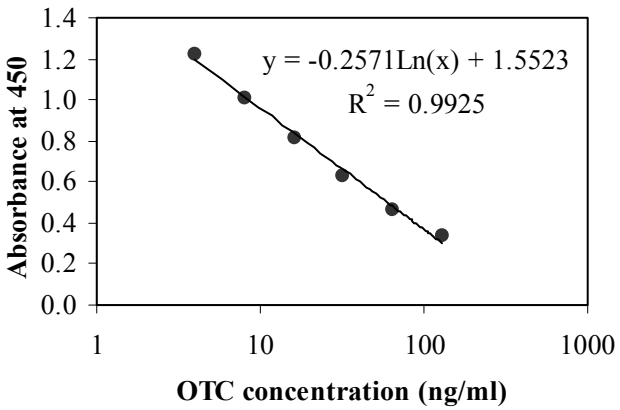


Figure 3. Standard curve of Ab-captured competitive direct ELISA with the detection range of 4-128 ng/ml

Table 2. Specificity of MAb

| Compounds | | %Cross reactivity |
|---|---|---|
| TCs group | Oxytetracycline (TC) | 100 |
| | Rolitetracycline (RTC) | 387 |
| | Chlortetracycline (CTC) | 6.6 |
| | Tetracycline (TC) | 1.6 |
| | Doxycycline | 0.16 |
| Other antibiotics | Norfloxacin | <0.01 |
| | Penicillin G | <0.01 |
| | Streptomycin | <0.01 |
| | Chloramphenicol | <0.01 |
| β-agonist | Clenbuterol | <0.01 |

## 4. Conclusions

The monoclonal antibody against oxytetracycline from hybridoma clone number 2-4F is sensitive enough to detect OTC at the current level of MRL. This MAb can be used in the development of an OTC test kit based on the antibody-captured competitive direct format.

## 5. Acknowledgements

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