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An Antibody-Based Qualitative Detection of Oxytetracycline Residues in Edible Fish Tissues

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Abstract In the present study we developed an Immunodot assay to detect oxytetracycline residue in fish edible tissues with a sensitivity as low as 60 ngL^{-1} of OTC. Anti-OTC rabbit antiserum was raised against OTC-BSA and used for assay development. The optimum conc. of coating antigen was 0.5 $mgmL^{-1}$. Random field samples were tested and obtained results were co-evaluated by HPLC to ensure the reliability. This report has presented a simple way to detect OTC residues in fish tissues.

Keywords Oxytetracycline, Immunodot, Anti-OTC rabbit antiserum.

Introduction

Aquaculture is one of the most important food producing sectors. Extensive use of antimicrobials in culture fisheries leads to the transit of drugs and their residues in food products intended for human consumption. It also causes to the release of drugs or their metabolites to the aquatic system [1]. Oxytetracycline (OTC), (Fig. 1) is a member of the broad-spectrum tetracycline (TC), approved by the US Food and Drug Administration (FDA) in catfish, salmon, and lobster to treat infections such as motile Aeromonas septicemia, Pseudomonas septicemia, and Enteric septicemia. Abuse of OTC in develops resistance upon exposure to antimicrobial agents and spread to humans by horizontal gene transfer [2] or resistant aquatic bacteria directly cause infections in humans [3]. Ultimately, this accumulation is likely to have serious health problems for human [4]. Therefore, several countries have set maximum residue limits (MRLs) for many food products [5]. European Union (EMEA/MRL/803/01–FINAL, 2001) has set the maximum residue limits (MRL) for OTC, 100 mg/kg in muscle, 300 mg/kg in liver and 600 mg/kg in kidney.

Existing chromatography methods, including high-performance liquid chromatography (HPLC) and liquid chromatography-tandem mass spectrometry (LC-ESI-MS/MS) and capillary electrophoresis have been used for the detection of TCs in food products [6—8]. Although these methods provide quantitative and accurate detection of OTCs but required expensive instruments, tiresome sample extraction procedures and expertise. Moreover, antibody based detections have been used owing to simplicity and cost-effectiveness with high sensitivity and specificity [9].

The aim of this study is to develop a reliable and simple method for the determination of oxytetracycline residues in edible fish tissues to ensure confidence in the animal productions and to limit the improper uses of veterinary drugs.

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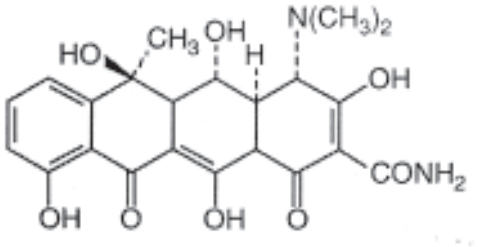

Fig. 1. Chemical structure of Oxytetracycline.

Table 1. Cross-reactivity studies of anti-OTC rabbit antiserum (pAb).

Antibiotics	IC_{50} (µg/1)	CR (%)
Oxytetracycline	7.76	100
4 epitetracycline	9.54	57
Tetracycline	132	5.23
Chlortetracycline	176	7.90
Minocycline	567	2.12
4-epi-tetracycline	678	3.23
4-epi chlortetracycline	764	2.02
Sulfadimethoxine	>10000	<0.01
Sulfamonomethoxine	>10000	<0.01

Materials and Methods

Preparation of artificial antigens

Artificial antigens were prepared according to [10] by Mannich reaction with slight modifications. Briefly OTC was dissolved in water : ethanol (2:1) then added to a solution containing BSA in ddH_2O. This was added to a solution of 3 M sodium acetate (pH 5.5) and 7.5% w/v formaldehyde at RT. Coating antigen (OTC-OVA) was prepared by the same method. Confirmation of Conjugates was done by SDS-PAGE.

Production of anti-OTC rabbit antiserum (polyclonal antibodies)

Two New Zealand white rabbits (6 weeks old, female) were injected (IM) with 800 µl of OTC–BSA (800 $\mu g ml^{-1}$) emulsified in Freund's complete Adjuvant (1:1). Remaining three booster doses were given with incomplete Freund's adjuvant on day 14, 28, and 35. Antiserum was collected and antibody titer checked by ELISA coated with OTC-OVA in 0.01M PBS. The antiserum was divided into 0.5 ml of aliquots and the hyper immune rabbit antiserum (PAb) was filtered through 0.45 µm syringe filter (Corning, USA) and purified by protein-A column (Bio-Rad, USA). Purified antiserum was stored at - 20^0C until use.

Cross - reactivity (CR) studies

The CR-studies determines the specificity of the antibody (anti-OTC rabbit antisera) and the reliability of the developed method. To determine the specificity, a set of several antibiotics were tested by icELISA according to [9]. Table 1 shows the results of CR tests.

Development of anti-OTC rabbit antisera based Immunodot

Immunodot was earlier developed in our laboratory earlier [11] for detection of WSSV. The method was adapted but modified as per the requirements here for detection of antibiotic residues. Briefly OTC–OVA (2–3µl) conjugate was dotted on to a nitrocellulose membrane (0.2µm Bio-Rad, USA) in different dilutions. PBS buffer was dotted as negative control. Dotted samples were air dried for 2 min and incubated with blocking buffer (3% Bovine serum albumin in 50 mM Phosphate buffer saline pH 7.4) for 30 min at room temperature (RT). The nitrocellulose membrane was washed 3 times using wash buffer (10mM PBS incorporated with 0.05% of tween 20) and reacted with anti-OTC rabbit antiserum at RT for 30 min. The membrane was washed with wash buffer and reacted with secondary antibody (1 : 1000 prepared in blocking buffer) for 20 min. The membrane washed again and treated with 4-chloro-1 napthol / H_2O_2 (Sigma, USA). Distinct precipitating purple dot at the site of antigen was considered positive.

Specificity testing

Specificity of Immunodot was checked using different tetracyclines and other antibiotics with the same method described above.

Fig. 2. Specificity of Immunodot.

Optimization of developed assay

Optimal concentration of coating antigen and antiserum titer was tested. The conc. of coating antigen (OTC–OVA) varied from 2mgmL^{-1} to 0.25 mgmL^{-1}.

Extraction of OTC from fish muscle samples

A rapid extraction method was followed according to [12]. Briefly 1g of muscle was minced, homogenized with 5 ml methanol with vigorus shaking for 5 min. Sample was directly used for Immunodot testing.

Table 2. Co-evaluation study with HPLC.

Sample no.	HPLC result (in ppb)	Immunodot result (in ppb)
S_1	13.432	–
S_2	67.002	+
S_3	0.000	–
S_4	10.971	–
S_5	2.324	–
S_6	2.006	–
S_7	3.342	–
S_8	0.000	–
S_9	0.000	–
S_{10}	120.221	+
S_{11}	0.000	–
S_{12}	0.000	–
S_{13}	0.000	–
S_{14}	2.002	–
S_{15}	0.000	–
S_{16}	0.000	–
S_{17}	0.000	–
S_{18}	78.002	+
S_{19}	2.785	–
S_{20}	0.000	–
S_{21}	0.000	–
S_{22}	104.026	+
S_{23}	0.000	–

Sample preparation for HPLC

The fish muscle was collected from randomly sampled fishes. OTC was extracted from the fish muscle samples according to [13].

Evaluation of the optimized Immunodot

A total number of 23 field samples were collected from different fish farms of Karnataka. Sample was extracted and tested by Immunodot. Obtained results were co-evaluated by HPLC to ensure the reliability.

Results and Discussion

Production of anti-OTC rabbit antiserum

Oxytetracycline is a small molecule with a molecular mass of 460.434 g/mol, non-immunogenic by its own. To make it immunogenic, it must be coupled to a carrier protein. Among protein carriers, BSA and OVA are two of the most commonly used ones, and usually, they give satisfying results. The A 280 result indicated the protein conc for OTC-BSA was 1.96 mg/mL and OTC–OVA was 1.24 mg/mL.

Cross–reactivity (CR) studies

The CR-studies determines the specificity of the anti-

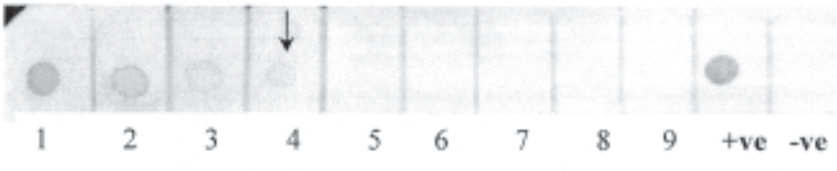

Fig. 3. Sensitivity of Immunodot at various conc of OTC ($ngml^{-1}$) (1) 500 ; (2) 250 ; (3) 125 ; (4) 60 ; (5) 30 ; (6) 15 ; (7) 7 ; (8) 4 ; (9) 2 ; (10) positive ; (11) negative.

body (anti-OTC rabbit antiserum) and the reliability of the developed method. To determine the specificity, a set of several antibiotics were tested for CR by ELISA (Table 1). The interference observed was negligible. The highest was obtained for OTC which showed a CR of 100%. For 4-epi OTC the CR was 57% might be due to the similar structure (reverse epimer of OTC). Thus, the developed immunoassay could be considered as a specific for OTC. A weak CR was also observed by structurally related compounds, such as TC (5.23%), CTC (7.90%), MC (2.12%), and 4-epiTC (3.23%), 4-epiCTC (2.02%), Sulfadimethoxine (<0.01%) and Sulfamonomethoxine (<0.01%). Therefore, it can be concluded that anti-OTC rabbit antiserum is highly specific. It could be implemented in developing assay.

Development of Immunodot

Different antibiotics were used for the confirmation of the specificity. Obtained result showed highest reactivity for OTC (98%), 4-epi Oxytetracycline (57%) but for other tetracyclines the cross - reactivity was below 8% which could be negligible. Cross-reactivity for other group of antibiotics found with no reaction (Fig. 2).

Optimization of Immunodot

Optimal concentration of coating antigen and antiserum titer

The antiserum titer was determined by non-competitive ELISA and the value was 1:32,000 with optimal concentration of coating antigen set at 0.5 mg/ml. So optimal concentration of the coating antigen OTC-OVA (0.5 mg/ml) and antiserum titer (1:32,000) were selected for further development.

Reaction time, sensitivity and specificity

Immunodot needed 90 min to complete the process except 5 min sample preparation time. The sensitivity was as low as 60 $ngmL^{-1}$ of OTC (Fig. 3). Anti-OTC rabbit antiserum was strongly reacting against OTC and 4-epioxytetracycline (Table 2).

Field analysis

A total number of 23 field samples were analyzed by Immunodot. Results were co-evaluated with HPLC results (Table 2). Report indicated that four samples contained OTC more than 60 $ngmL^{-1}$ whereas only two samples contained OTC more than the MRL (100 ppb) level. Remaining nineteen samples were safe to consume. The co-evalutionary studies exhibited the reliability of developed technique.

Conclusion

In this work, we developed an enzyme based Immunodot enables the qualitative detection of Oxytetracycline residues in edible fish tissues. This assay can detect up to 60 $ngmL^{-1}$ of OTC. The sensitivity is below MRL level. Immunodot was successfully

tested on different field with specificity. Therefore, this simple and inexpensive method easily made OTC analysis one step closer to reality.

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