Lateral Flow Immunochromatograpphic Assay (LFIA) for the Detection of Tetracycline

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ABSTRACT

In this study, lateral flow immunochromatograpphic assay (LFIA) was developed to detection tetracycline (TC). The LFIA was based on competitive format between TC conjugated with protein carrier and TC in samples. A monoclonal antibody against tetracycline (MAb-TC) was produced by hybridoma cell culture. Goat anti mouse (GAM) and TC conjugated with bovine serum albumin (TC-BSA) were prepared and immobilized on an analytical membrane at the control line and test line, respectively. Colloidal gold particles with the averaged size of 40 nm were attached to the MAb-TC and applied on to conjugate pad. The test result could be observed within 10-15 min by naked eyes. The visual limit of detection (VLOD) was found to be 20 ng mL^{-1} and the developed LFIA showed no cross-reactivity with oxytetracycline and chlortetracycline.

Keywords: Immunochromatographic assay, Test strip, Tetracycline

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Introduction

Tetracycline (TC) is a broad spectrum antibiotic in the tetracyclines (TCs) group. It is widely used for prevention and control of infectious diseases against a variety of Gram-positive and Gram-negative bacteria. Several countries have set maximum residue limits (MRLs) for TCs. In the USA, the MRLs of TC in muscle, liver and kidney are 2, 6 and 12 mg kg^{-1}, respectively. The European Commission set MRLs of TCs (i.e., Tetracycline : TC, Oxytetracycline : OTC, Chlortetracycline : CTC and their 4-epimers) in muscle, liver, kidney and milk at 200, 600, 1200 and 100 µg kg^{-1}, respectively (Codex alimentarius, 2012; Official Journal of the European Union, 2010). However, long term use and misuse of TC can cause problem of drug residues in animal products. This affects not only the quality of products but also the health of consumers. Therefore, monitoring of TC residues is mandatory.

Detection method for TCs and other antibiotic residues can generally be divided into two methods, which are confirmatory method and screening method. The confirmatory method is mostly based on high performance liquid chromatography (HPLC) (Nebota *et al.*, 2014) and liquid chromatography mass spectroscopy (LC-MS) (Venkates *et al.*, 2013). These methods are useful for quantitative analysis which yield very high accuracy and precision result. However, these methods are time-consuming and require expensive laboratory equipment and well trained personnel. On the other hands, the screening method such as enzyme-linked immunosorbent assay (ELISA) and lateral flow immunoassay (LFIA) are very useful for qualitative and semi-quantitative analysis. These methods are relatively inexpensive and suitable for multi sample analysis (Cháfer-Pericás *et al.*, 2010). LFIA or immunochromatographic assay or strip test is widely use as a on-site detection tool because of its one-step analysis, visual assessment by naked eyes, high throughput, easy to use and short analysis time. LFIA is an increasingly popular tool for detecting antibiotic residues including TCs such as oxytetracycline, chlortetracycline and tetracycline (Chen *et al.*, 2016; Alfredsson *et al.*, 2005) and doxycycline (Le *et al.*, 2011). The format of LFIA can be divided into two types depending on the mass of the analyte. Sandwich assay format is usually used for the analyte with high molecular mass while the competitive assay format is used for the analyte with low molecular mass (Posthuma-Trumpie *et al.*, 2008).

Objective of the study

To develop a lateral flow immunochromatograpphic assay (LFIA) for tetracycline residues detection in food samples.

Materials and Methods

Material and reagent

Tetracycline hydrochloride (TC-HCl), oxytetracycline hydrochloride (OTC-HCl), chlortetracycline hydrochloride (CTC-HCl) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, USA). BSA Protein Assay kit was obtained from Thermo scientific (Rockford, Illinois). Hitrap Protein G affinity HP was purchased from GE Healthcare (Sweden). Formaldehyde was obtained from Carlo erba (France). Goat anti mouse (GAM) were purchased from Jackson Immuno (West Grove, PA, USA). Colloidal gold 40 nm was obtained from Kestrel Biosciences Co., Ltd. (Pathumthani, Thailand). ISF-1 serum free media was received from Biochrom GmbH

(Berlin, Germany). Sample pad (bound glass fiber, STANDARA 17), analytical membrane (nitrocellulose membrane, AE 99), absorbent pad (100% cotton linter, CF 7) and conjugate pad (bound glass fiber, GF 33) were obtained from Whatman (Kent, USA).

Purification of monoclonal antibody against TC (MAb-TC)

The hybridoma cells producing monoclonal antibody against TC no. 12-3F were thawed at 37 ºC and then cultured in ISF-1 medium at 37 ºC in 5% CO_2 incubator. The culture medium was collected by centrifuged at 16,000 x g for 30 min and the MAb in the supernatant was purified by Protein G affinity chromatography. The Protein G affinity column was equilibrated with 2mM phosphate buffer, pH 7.0 at flow rate 2.0 mL min^{-1}. After loading and washing step, the bound MAb was eluted with 0.1M glycine-HCl, pH 2.7 and fractionally collected in 60 µl of 1M Tris-HCl, pH 9.0 to neutralize the pH. The protein concentration of each fraction was quantified by measuring the absorbent at 280 nm. The fractions with high protein concentration were pooled together and stored at -20 ºC until use (Tesvichian, 2010).

Preparation of monoclonal antibody labeled with colloidal gold (MAb-Colloidal gold)

The optimum ratio of MAb to colloidal gold particles was studied. MAb-TC (20 µL) at various concentrations (0 to 1 mg mL^{-1}) prepared in 2mM sodium borate buffer, pH 8.2 was separately added to the colloidal gold solution (200 µL) in 2mM sodium borate buffer, pH 8.2. The mixtures were incubated for 1h at room temperature. Then, 10 % NaCl (80 µL) was added and the absorbance of the solution was measured at 520 nm. The optimum concentration of MAb-TC for labelling with colloidal gold particle was the least concentration that gives the highest absorbance value (Chen *et al*., 2016).

MAb-TC solution (1 mL) at optimum concentration was added to the colloidal gold solution (10 mL) and incubated under stirring for 1 h at room temperature. Then, 5% (w/v) BSA solution (1 mL) was added drop wised. The mixture was incubated for 1h at room temperature. The conjugate was collected by centrifugation at 16,000 x g for 30 min, 4 ºC and resuspended in 1 mL of 2mM sodium borate buffer, pH 8.2 containing 2% sucrose. The MAb-colloidal gold conjugate was stored at 4 ^{o}C until use cassette (Zhang *et al*., 2006).

Preparation of conjugate pad

The conjugate pad (GF 33) with the size of 4 mm x 10 mm was treated with 1% (w/v) BSA in 0.1% (v/v) tween-20 in phosphate buffer (containing 2% sucrose) for 15 min. The conjugate pad was then dried for 1h at 37 ºC. The MAb-colloidal gold solution (2 µL) at the dilution of 1/1, 1/2, 1/3 and 1/4 was applied at the center of the conjugate pad and the pads were stored in a desiccator at room temperature until use.

Preparation of analytical membrane

Nitrocellulose (NC) membrane (size 4 mm x 25 mm) was used for analytical membrane which consists of test line (T-line) and control line (C-line). An optimized concentration and volume of TC-BSA conjugate was sprayed onto NC membrane at the T-line. TC-BSA conjugate was prepared based on Mannich reaction. Briefly, 10 mg of BSA dissolved in 1 mL of 0.1M MES buffer (containing with 0.15 M NaCl), pH 4.7 was mixed with 1mL of 10 mg mL^{-1} TC solution. Then 250 µL of 37% (v/v) formaldehyde was added into the TC-BSA solution. The solution mixture was incubated in the dark at room temperature overnight and then dialyzed against phosphate buffer saline,

pH 7.4 for 5 times (Hermanson, 1996). The goat anti mouse IgG (GAM) at 0.5 mg mL^{-1} was sprayed onto NC membrane as the C-line. The T-line and C-line were sprayed at a flow rate of 1µL cm^{-1} using the IsoFlo Flatbed Dispenser (Imagine technology, USA). After that the NC membrane was dried for 15 min at room temperature and treated with 1% (w/v) BSA in phosphate buffer, pH 7.2 for 15 min and dried at 37 ºC for 1h in order to reduce non-specific binding in other areas outside the T- and C-line. The sprayed NC membrane was attached on the center of plastic backing card (length 30 cm) and cut into each 4 mm wide test strip.

Preparation of sample pad and absorbent pad

Sample pad and absorbent pad were cut in size of 4 mm x 10 mm and dried at 37 ºC for 1h before use.

Assembly of LFIA test strip

Test strip consists of four sections: sample pad, conjugate pad, analytical NC membrane and absorbent pad which are attached on a plastic backing card as shown in figure 1. The test strip was placed in a plastic.

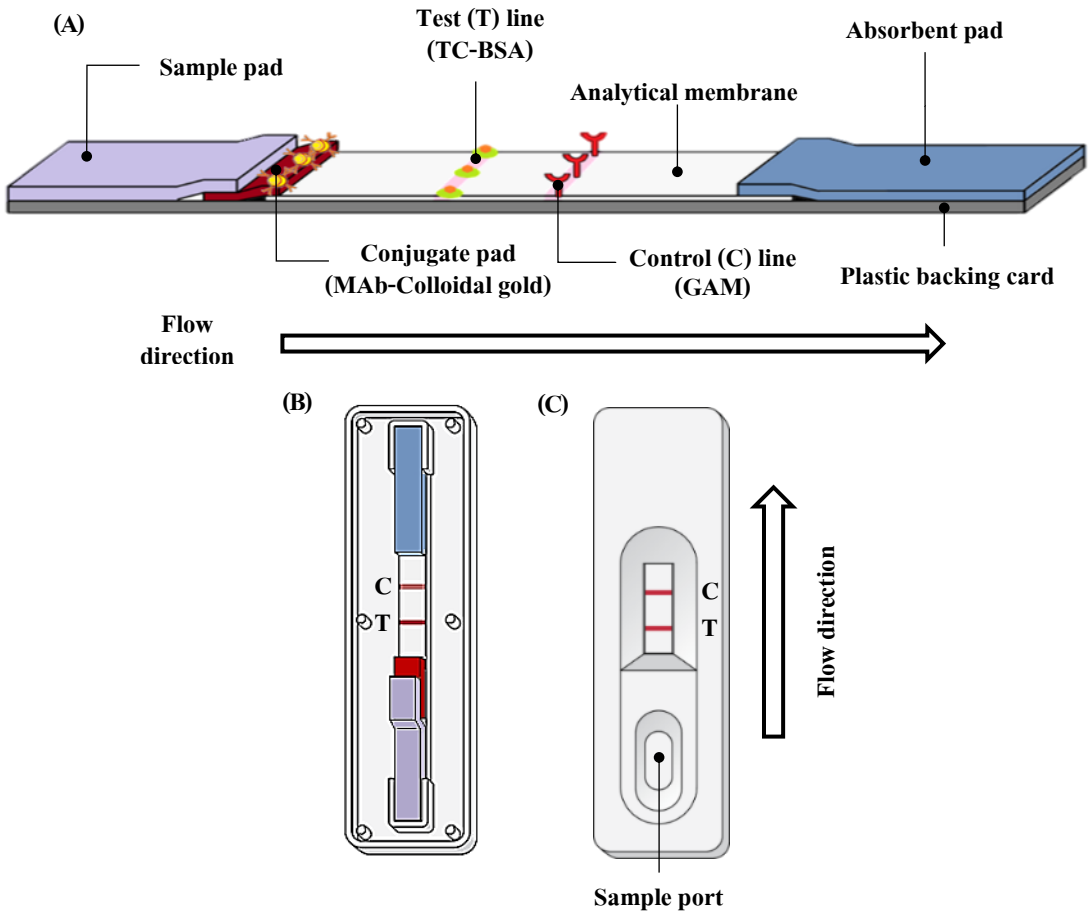


Figure 1 Schematic diagram of a LFIA test strip. (A) overlapping layout of four sections: sample pad, conjugate pad (MAb-Colloidal gold), analytical membrane (T-line, TC-BSA and C-line, GAM) and absorbent pad, (B) placement of test strip in a plastic cassette. (C) completely assembling test strip.

Test procedure

Sample (100 µL) was loaded onto the sample pad at the sample port. During the movement of sample solution passed thru the conjugate pad due to capillary force, TC presented in the sample bound to the MAb-Colloidal gold. Both the TC- MAb-Colloidal gold complex and the remaining unbound MAb-Colloidal gold move pass the test line to the control line where the remaining unbound MAb-Colloidal gold can bind to the goat anti-mouse antibody. As a result, the purple color of colloidal gold particle could be observed only at the control line. On the contrary, if there was no analyte in the sample, the MAb-Colloidal gold moved along with the sample solution to bind with TC-BSA at the test line and the remaining unbound MAb-Colloidal gold can bind to the goat anti-mouse antibody. Consequently, the purple color of colloidal gold particle could be observed at both the test line and the control line. The color intensity at the test line varied inversely with the amount of TC in the sample. If purple color could not be observed at the color line, the test was invalid or incorrect as shown in figure 2. (Liu *et al*., 2014).

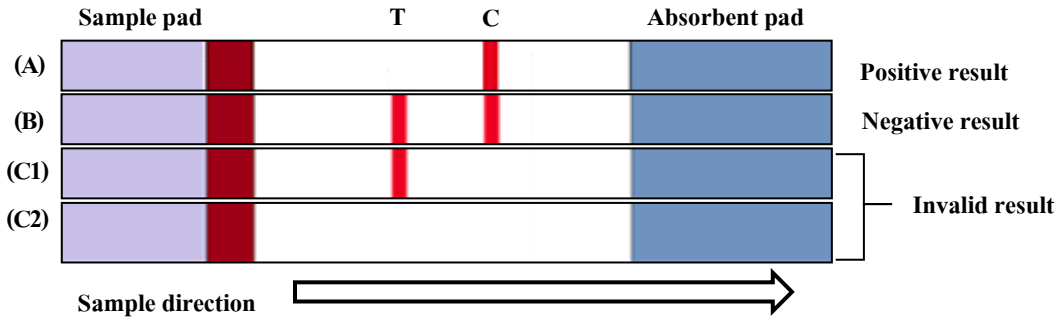


Figure 2 Interpretation of LFIA test strip for detection of TC. (A) Positive n result, (B) Negative result and (C1-C2) Invalid result.

Sensitivity and specificity of LFIA test strip

The sensitivity of LFIA test strip was determined by testing with the TC standard solution at 0-80 ng mL^{-1}. The visual limit of detection (VLOD) by naked eyes was defined as the least concentration of TC solution giving no color at the T-line of the strip.

The specificity of LFIA test strip was determined by the cross-reaction with OTC and CTC at 0-80 ng mL^{-1} (Chen *et al*., 2016).

Results

Optimization of LFIA test strip

Firstly, the optimum ratio of the colloidal gold particle to the MAb was investigated. The absorbances of the MAb-gold particle solution prepared by using different concentrations of MAb were shown in figure 3. It was found that the lowest concentration of MAb-TC which gave the highest absorbance value was 62.5 µg mL^{-1}.

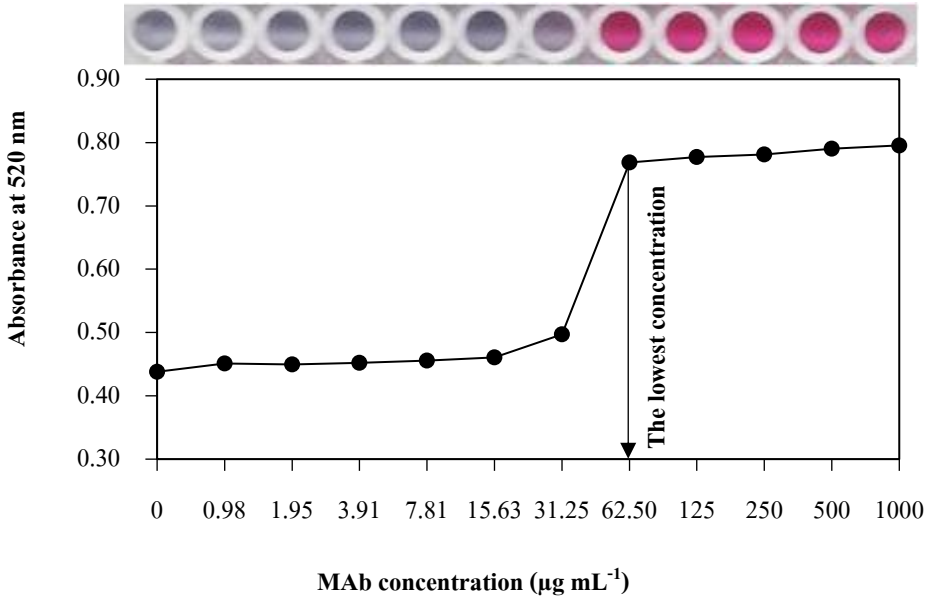


Figure 3 Absorbance values at 520 nm of the MAb-Gold particle solution prepared at different concentrations of the MAb.

To find the optimum concentration of TC-BSA which should be immobilized at the T-line, different concentrations (0.25, 0.5, 0.75, 1.0 and 1.25 mg mL^{-1}) of TC-BSA were sprayed while a fixed concentration of GAM at 0.5 mg mL^{-1} was sprayed at the C-line position. The analysis using buffer solution as the sample showed that the color intensity of the T-line were higher than the C-line at 1.25 and 0.75 mg mL^{-1} and was decreased at 0.5 mg mL^{-1}. Until at 0. 25 mg mL^{-1}, the color intensity of the T-line and the C-line were comparatively in the same level. Therefore, the optimum of T-line was immobilized at 0.25 mg mL^{-1} of TC-BSA was used (figure 4).

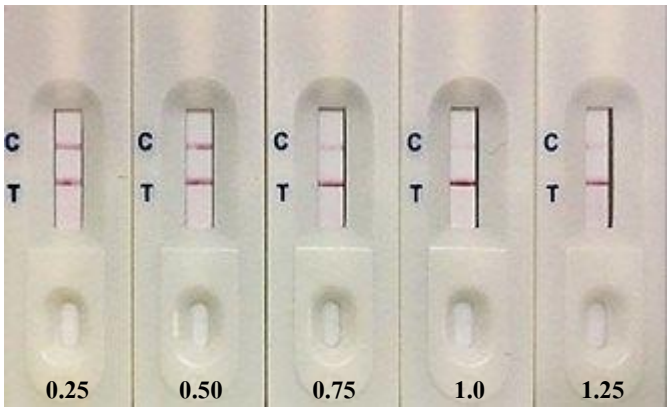


Figure 4 Color intensity of the T-line and C-line of the test strip prepared at different concentrations of TC-BSA.

Another parameter of the LFIA test strip for optimization in this study was the dilution of MAb-colloidal gold that applied on the conjugate pad. The result shown in the figure 5. When the dilution of MAb-colloidal gold was increased, the color intensity of T-line and C-line was decreased. In this study the dilution at 1/3 gave a sufficient color intensity of T-line and C-line.

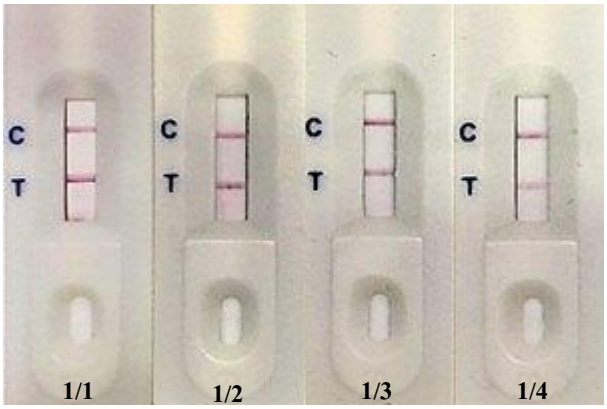


Figure 5 Color intensity of the T-line and C-line of the test strip prepared at different dilutions of the MAb- gold conjugate (C-line: GAM 0.5 mg mL^{-1}, T-line: TC-BSA 0.25 mg mL^{-1}).

Sensitivity and specificity of LFIA test strip

The LFIA test strip was prepared with the optimum condition. The T-line and C-line were immobilized with 0.25 mg mL^{-1} of TC-BSA and 0.5 mg mL^{-1} of GAM, respectively. The conjugate pad was applied with MAb-colloidal gold at the optimum dilution of 1/3. Under this condition, the assay sensitivity was investigated with TC standard at the concentration range of 0-80 ng mL^{-1}. The result shown in figure 6. indicated that the color intensity of the T-line varied inversely with the TC concentrations and started to completely disappear when 20 ng mL^{-1} of TC was used. Therefore, the visual limit of detection of this test strip was defined at 20 ng mL^{-1}.

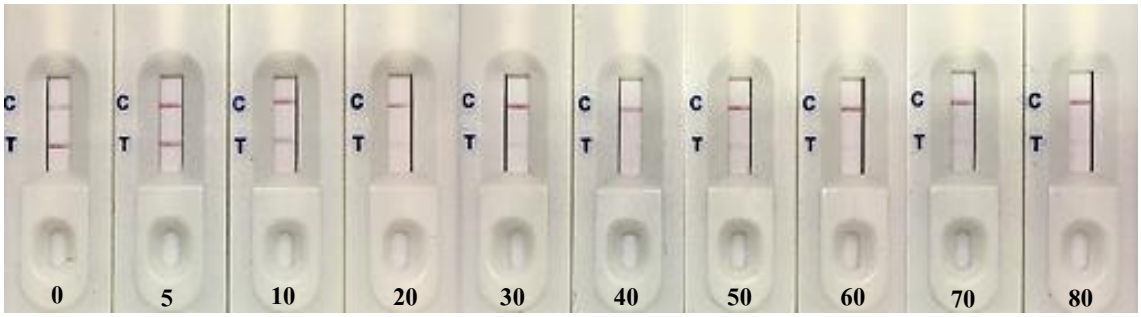
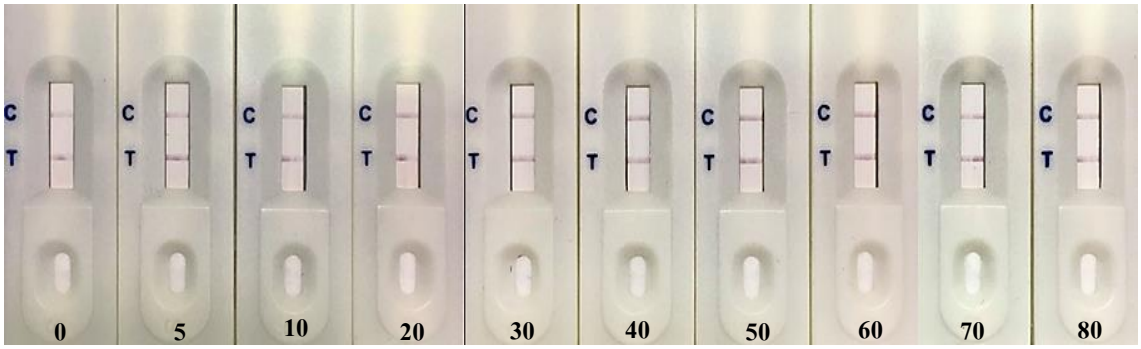
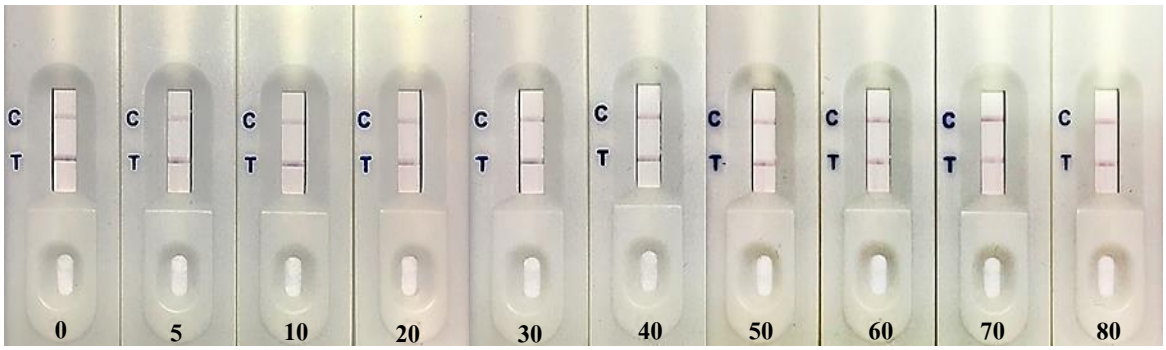


Figure 6 Color intensity of the T-line of the developed test strip at different concentrations of TC standard (0, 5, 10, 20, 30, 40, 50, 60, 70 and 80 ng mL^{-1}).

In addition, the test strips were used to detect OTC and CTC in order to check for their specificity. As shown in the figure 7, change in color intensity at the T-line was not clearly noticeable when either OTC or CTC standard were used as the sample. This result suggested that there was no cross-reactivity with OTC and CTC.



OTC concentration (ng mL^{-1})



CTC concentration (ng mL^{-1})

Figure 7 Cross reactivity of LFIA test strip with OTC and CTC.

Discussion

TC has a molecular weight of 444.44 Da which is considered to be a small molecule. Therefore, LFIA test strip based on a competitive format was developed. In this study, a suitable amount of colloidal gold adsorbed on to the MAb can prevent the coagulation of MAb labeled with colloidal gold, so the concentration of the MAb must be optimized. The optimum concentration of TC-BSA and MAb-Colloidal gold were important, because the amount of TC-BSA and MAb-Colloidal gold was directly affect the sensitivity of the LFIA test strip. The VLOD was found at 20 ng mL^{-1} and no cross reactivity with OTC and CTC was observed. Previously, LFIA using polyclonal antibodies against TC was developed to detect TC, OTC and CTC in milk honey and animal body fluids with higher sensitivity with the LOD in the range of 60 pg mL^{-1} to 10 ng mL^{-1} (Taranova *et al.*, 2015). In another study reported by Chen *et al.* (2016), LFIA was developed with the obtained VLOD in the range 15-40 ng mL^{-1}. Even though, the test strips

developed in our study were not highly sensitive as compared to those reported, they could still be used to detect TC because the obtained VLOD was lower than the currently regulated MRL level (100-1200 μg kg^{-1}).

Conclusions

Under the optimum preparation condition, the VLOD of the developed test strip was found at 20 ng mL^{-1} of TC and no cross reactivity with OTC and CTC was observed. The developed test strip is sensitive enough to detect TC at the current level of MRL and can be applied to detect TC residues in food sample such as honey and chicken.

Acknowledgements

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