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Conjugation of tetracycline with carrier proteins and production of its polyclonal antibody for the development of rapid test

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Abstract. Tetracycline (TC) is a broad-spectrum antibiotic used in the treatment of several infectious diseases. The excessive use of tetracycline antibiotics, especially tetracyclines in livestock, has raised concerns about residues of tetracycline antibiotics in food that may affect consumers' health. It is necessary to develop a rapid test strip for detecting tetracycline antibiotic residues in food and animal feed. This paper presents the suitable conditions for the conjugation of tetracycline to BSA or KLH: molar ratio of TC:BSA/KLH was 25:1, formaldehyde concentration of 5 %, temperature of 4^{o}C and reaction time of 4 hours. The KLH-TC conjugate was used to successfully raise polyclonal antibodies against TC in rabbits. The anti-TC polyclonal antibodies were purified and could be used for the development of the lateral flow immunoassay test strip for the detection of tetracycline.

Keywords: tetracycline, conjugation, polyclonal antibody, rapid test.

Classification numbers: 1.3.2, 2.10.3, 3.7.2.

1. INTRODUCTION

Tetracycline is a group of broad-spectrum antibiotics used for medical as well as livestock purposes. Several properties make tetracyclines the most widely used of all antibiotics: active againsta wide range of Gram-positive and Gram-negative bacteria, well absorbed orally, low toxicity and side effects, and relatively cheap. Therefore, tetracycline is widely used forthe treatment and prophylactic control of bacterial infections in humans and animals[1, 2]. The antibiotic tetracycline is also used as a growth promoter to improve growth performance in animals. For these reasons, the antibiotic tetracycline is an extremely popular veterinary antibiotic.This has raised concerns because the presence of tetracycline residues in foods may affect human health and increase microbial resistance. Many countries around the world, including Vietnam, have established maximum residue levels (MRLs) of antibiotics to protect

consumers. The maximum residue limit of tetracycline in food in Vietnam is specified in the National Standard TCVN 6711:2010 (CAC/MRL 2-2009).

Residues of tetracycline antibiotics in food can damage the liver and kidneys, cause allergic reactions, upset stomachs in consumers, and possibly cause antibiotic resistance [2]. Furthermore, the excessive use of tetracycline in husbandry has also attracted significant attention from consumers and affected the acceptance of our livestock products when exported to other countries.

Tetracycline residue can be detected using a variety of methods such as microbiological techniques, liquid chromatography-mass spectrometry (LC-MS), high-performance liquid chromatography (HPLC), Enzyme-Linked Immunosorbent Assay (ELISA), and lateral flow immunoassay (LFA) [3-6]. Besides the lateral flow immunoassay method, other methods are complicated, time-consuming, well-equipped laboratories, and require trained technicians. Therefore, they are not suitable to be used for a large quantity of samples and on-site applications.

Lateral flow immunoassay is an immunological method known as a rapid, simple, sensitive, specific, and cheap technique. The sensitivity and specificity of this method are based on the specific antibodies used. Tetracycline is a small molecule that is unable to elicit an immune response by itself to produce specific antibodies.

Conjugation of tetracycline or its derivatives with carrier proteins has been performed using various methods. Antibiotic-carrier conjugate and its antibodies have also been used to develop detection methods such as ELISA and rapid test strips [7 - 9]. However, the study of the conjugation of tetracycline with two carrier proteins (Bovine serum albumin - BSA and Keyhole Limpet Hemocyanin - KLH) and its polyclonal antibody for lateral flow assay application has not been studied. Therefore, the aim of this study was to determine the appropriate conditions for TC conjugation with carrier proteins (BSA and KLH) and produce tetracycline-specific antibodies for rapid test kit development.

2. MATERIALS AND METHODS

2.1. Materials

Tetracycline (TC), Keyhole Limpet Hemocyanin (KLH), Goat anti-rabbit IgG antibody (H+L) HRP conjugate, gold nanoparticles were purchased from Sigma (USA). Formaldehyde;3, 3’5, 5’-tetramethylbenzidine (TMB) liquid substrate for ELISA, Bovine serum albumin (BSA) were purchased from Merck(USA). Protein A sepharose bead was purchased from ThermoFisher Scientific(USA).Other chemicals and solvents were purchased from Merck and Sigma-Aldrich. Complete Freund’s adjuvant (CFA) and Incomplete Freund’s adjuvant (IFA) were purchased from BD (USA). Materials for the test strip preparation including cellulose membrane, sample pad, conjugate pad and adsorption pad were purchased from Merck-Millipore (USA). Specific pathogen free (SPF) rabbits at 3 months old were supported by Center for Research and Production of Vaccines and Biologicals - POLYVAC (Viet Nam).

2.2. Conjugation of tetracycline with carrier proteins

Tetracycline (TC) and carrier proteins (BSA, KLH) were coupled via a mannish reaction under the catalysing of formaldehyde [7]. The conjugates BSA-TC and KLH-TC were prepared by mixing BSA or KLH with tetracycline and formaldehyde at a final concentration of 0.1 mM,

2.5 mM, and 5 %, respectively. The mixture was incubated at 4 ^{o}C for 4 hours under light-protected conditions and gentle shaking. The conjugate (BSA-TC or KLH-TC) was then washed five times with 1X PBS, pH 7.4using Amicon Ultra-15 centrifugal filter, 10 kDa. The conjugate solution was scanned by UV/Vis spectrophotometerwith a wavelength range of 250 - 600 nm to determine the quality of the BSA-tetracycline/KLH-tetracycline conjugate. The obtained solution was stored at -20 ^{o}C for further studies.

2.3. Production of polyclonal antibodies agains ttetracycline in rabbits

Two healthy, specific pathogen-free rabbits were isolated separately and examined ten days before the injection. Rabbits were immunized with the KLH-TC conjugate as follows: 250 µL of KLH-TC conjugate (1 mg/mL) was emulsified with an equal volume of Complete Freund's adjuvant for the first injection and Incomplete Freund's adjuvant for the booster injections at 2-week intervals. The first injection was performed intradermally, and the booster injections were introduced intramuscularly. Before each injection, blood was obtained by bleeding the central auricular artery of the rabbit's ear and antibody titers were determined by an indirect ELISA. Rabbit blood was completely collected 2 weeks after the final injection. Antiserum was separated from the clot by the decant method. The antibody was purified using Protein A sepharose bead column as the instruction of the producer. The purity of antibody was determined by SDS-PAGE commassie brilliant blue staining and the ImageJ software.

2.4. Enzyme-linked immunosorbent assay (ELISA)

The procedure was carried out as described in the previous study [8] with some adjustments. Each well was coated with the optimal dilution of BSA-TC conjugate in 50 mM carbonate buffer, pH 9.5 overnight at 4 °C. After washing three times with PBS containing 0.05 % Tween 20 (PBS-T), nonspecific binding siteson the well surface were blocked with PBS containing 5 % non-fat dry milk for 2 hours, followed by two washes with PBS-T. Primary antibodies (rabbit antiserum, purified rabbit anti-tetracycline antibody) were added to each well and incubated for 2 hours at 37 ^{o}C. The plate was washed 5 times with PBS-T and incubated with the secondary antibody conjugated with HRP for 1 hour at room temperature. After washing five times, the TMB substrate solution was added to each well. After 20 minutes of incubation in a dark room, the reaction was stopped with a 5 % solution of sulfuric acid. The absorbance of wells was read by a plate reader at 450 nm.

2.5. The design of the lateral flow immunoassay (LFA) test strip for the detection of tetracycline

In this study, the LFA test strip was designed as described by the previous studies [9, 10]. The LFA strip consists of four sections: a sample pad, a conjugate pad (containing anti-tetracycline antibodies – gold nanoparticles conjugate), a nitrocellulose membrane, and an absorbent pad. The TC-BSA conjugate was immobilized to the membrane at the test site (T-line), and protein A was immobilized on the test strip at the control position (C-line). The conjugate pad contains a conjugate complex between prepared anti-TC antibodies and gold nanoparticles. The sample solution was loaded onto the test strip at the loading sample window. When added to the sample pad, the sample quickly spreads toward the nitrocellulose membrane due to capillary action. If the sample is tetracycline-negative, the anti-tetracycline antibodies – gold nanoparticles conjugate will be free and captured by tetracycline-BSA and Protein A

immobilized at the T-line and C-line positions, resulting in the appreance of two signal bands.If tetracycline is present in the sample (TC-positive), tetracycline will bind to the anti-tetracycline antibodies – gold nanoparticles conjugate and prevent this conjugatefrom binding to tetracycline-BSA immobilized at the T-line position, resulting in the presence of only one colored band in the control line.

3. RESULTS AND DISCUSSION

3.1. Tetracycline – bovine serum albumin conjugation

Tetracycline (TC) is a low molecular weight compound (444.435 g/mol) and is considered as a hapten. Therefore, in order to recognize this antibiotic by a specific antibody, TChas to be covalently conjugated to a carrier protein. Commonly, BSA and KLH are used for conjugation. In this study, TCs have been coupled to carrier protein via the Mannich reaction, as shown in Fig. 1. In the Mannich reaction, an amine group of protein is coupled to a suitable carbon atom in the hydrocarbon rings of tetracycline.

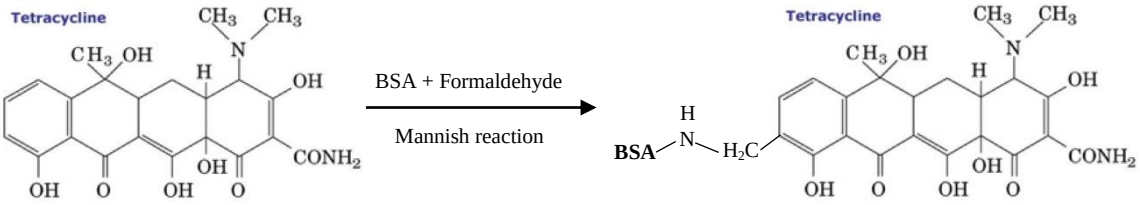

Figure 1. Reaction scheme of BSA-TC synthesis. [9]

3.1.1. Establishment of initial condition for conjugation of bovine serum albumin and tetracycline

In this study, conjugation of TC to BSA was carried out at initial conditions of 0.1 mM BSA, 2.5 mM tetracycline, and under thecatalyzing of formaldehyde for 4 hours. After five washes, the conjugate solution was scanned with a wavelength range of 250–500 nm to demonstrate whether the conjugation was successful. The absorbance curves are shown in Fig. 2. The result showed the appearance of two peaks at 274 nm and 371 nm. These two peaks had a displacement relative to the two peaks of TC (269 nm and 364 nm).

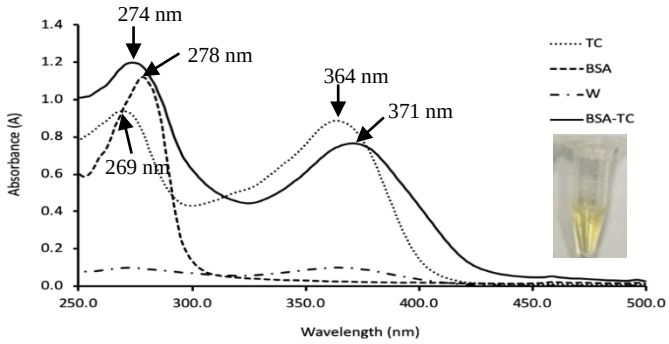

Figure 2. Spectral scanning results of tetracyline, bovine serum albumin and BSA-TC conjugate. TC, tetracycline; BSA, bovine serum albumin; W, final wash fraction; BSA-TC, BSA-TC conjugate.

The peak of 274 nmwasthe middle of BSA's peak (278 nm) and the first peak of TC (269 nm). This peak shift after conjugation with carrier proteins was also observed in the previous studies [7, 9, 11]. The obtained product hadthe characteristic yellow color of tetracycline (Fig. 2).The obtained results revealed that tetracyline was successfully conjugated to BSA.

3.1.2. Determination of themolar ratio of tetracycline and BSA

The concentration of TC in the solution has an important influence on the success of the conjugation and resulting in coupling efficiency. Theoretically, one BSA molecule can bind up to 30 - 40 TC molecules [7]. Therefore, a range of the concentration ratios TC: BSA of 30:1; 25:1; 20:1; 15:1, and 10:1 were investigated in this study to determine optimal ratios for conjugation. The final concentration of BSA in the mixture was 0.1 mM, therefore the final concentration of TC in the reaction was 1.0; 1.5; 2.0; 2.5 and 3.0 mM. The obtained results showed that the absorption intensity of the solution increased with increasing TC concentration.Spectral scanning results showed that the first peak tended to shift from the maximum absorption wavelength of BSA protein (278 nm) to the maximum absorption wavelength of TC (269 nm) when increasing TC concentration (Fig. 3a).These results meant that many more TC molecules were attached to the BSA carrier . However, the results also showed that protein precipitation appeared when using TC concentration at 3 mM, while at other concentrations, this phenomenon was not observed (Fig. 3b). Chen *et al.* conjugated TC with BSA at a molar ratio of 47/1 [9]. From the obtained results, the TC/BSA concentration ratio of 25/1 was selected for further studies.

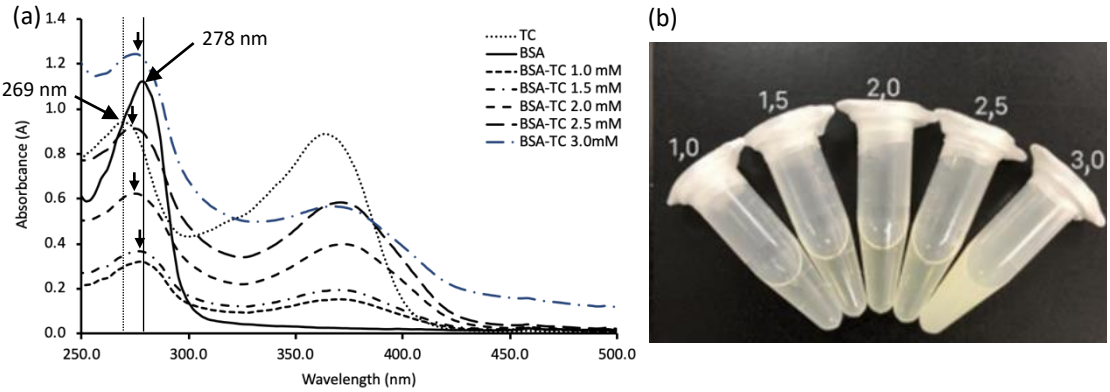

Figure 3. Spectral scanning results (a) and image of BSA-TC conjugate solutions (b) at different concentrations of tetracycline (1.0, 1.5, 2.0, 2.5 and 3.0 mM). TC, tetracycline; BSA, bovine serum albumin; BSA-TC, BSA-TC conjugate.

3.1.3. Determination of the formaldehyde concentration

In the conjugation reaction between TC and BSA, formaldehyde acts as both a catalyst and a reactant to form the bridge between TC and BSA. According to previous studies, the concentration of formaldehyde used was from 3 % to 6 % [7, 9]. In this study, a concentration range of formaldehyde of 0 %, 1 %, 2 %, 3 %, 4 %, and 5 % was investigated. Spectral scanning results showed that all samples had two peaks in the presence of formaldehyde, while there was only one peak for samples without formaldehyde (Fig. 4).The obtained result indicated that TC was not bound to the BSA in the absence of formaldehyde and resulted in only a specific peak of protein on the scanning spectrum.The results also showed that the absorption intensity increased

with an increasing formaldehyde concentration in the reaction.In addition, the maximum absorption wavelength at the first peak also tended to shift from the protein peak (278 nm) towards the formaldehyde peak (269 nm). These observationsdemonstrated that more TC molecules were attached to BSA molecules with an increasing concentration of formaldehyde. Based on the obtained results in this study, the concentration of formaldehyde used for further studies should be 5 %.

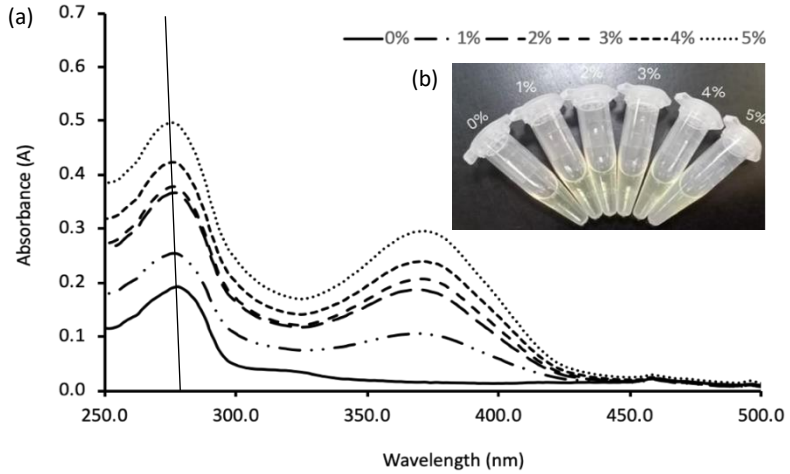

Figure 4. Spectral scanning results (a) and image of BSA-TC conjugate solutions (b) at different concentrations of formaldehyde (0, 1, 2, 3, 4 and 5 %).

3.1.4. Effect of reaction temperature and time

The Mannich reaction is very sensitive to the reaction temperature. The high temperature could improve the reaction rate and result in a shorter reaction time. However, the Mannich base is unstable at high temperatures and the reactant, such as formaldehyde oxidizes at elevated temperatures [12]. In addition, carrier protein (BSA) is also a temperature-sensitive component. Therefore, the effect of temperature was also investigated. The conjugation reaction was carried out at three temperature conditions of 4 ^{o}C, 25 ^{o}C and 37 ^{o}C.The results showed that the conjugation efficiency decreased at the elevated temperatures (25 ^{o}C and 37 ^{o}C), while the highest efficiency was obtained when the reaction was carried out at 4 ^{o}C (Fig. 5A).The conjugation reaction temperature of 4 ^{o}C was selected for further studies.

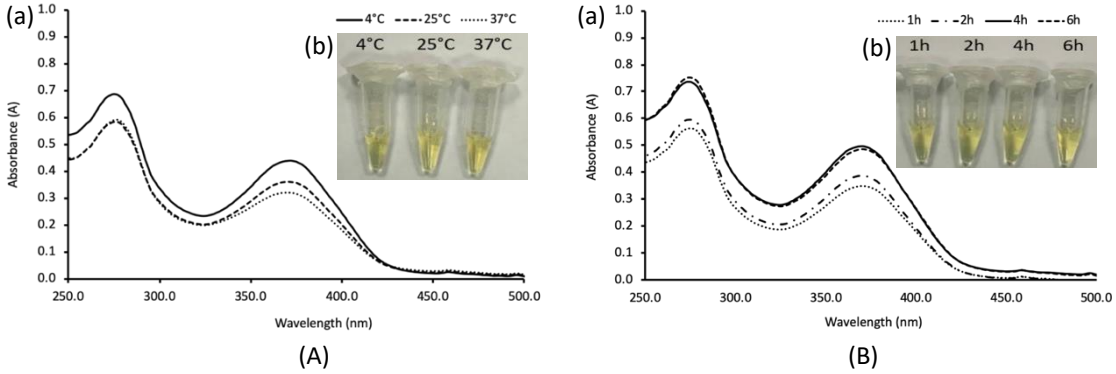

Figure 5. Spectral scanning results (a) and image of BSA-TC conjugate solutions (b) at the different conjugation reaction temperatures (4 ^{o}C, 25 ^{o}C and 37 ^{o}C) (A) and time (1, 2, 4, 6 hours) (B).

To select a suitable reaction time, the conjugation reaction was carried out for 1, 2, 4, and 6 hours at 4 ^{o}C in the dark condition. The results showed that the conjugation efficiency increased when the reaction time was increased from 1 hour to 4 hours. However, the conjugation efficiency did not increase when the reaction time was increased to 6 hours (Fig. 5B).

From obtained suitable conditions, the BSA-TC conjugate was prepared with a large amount for further application. The concentration of conjugate solution was approximately 10 mg/mL.

3.2. Tetracycline – Keyhole Limpet Hemocyanin conjugation

3.2.1. Determination of the molar ratio of tetracycline and KLH

In the study of Noraini *et al.* [7], the tetracycline was conjugated to KLH at a molar ratio of 46:1 under catalyzing by formaldehyde. The concentrations of tetracycline and KLH were 0.46 mM and 0.01 mM, respectively. In another study, the oxytetracycline was conjugated to KLH at a molar ratio of 4340:1 [8]. In this study, a range of the concentration ratios TC: KLH of 30:1; 25:1; 20:1; 15:1, and 10:1 were investigated. The concentrations of tetracycline in the reaction were varied from 0 mM to 3 mM. The results showed that the conjugation efficiency was increased when the molar ratio of TC:KLH was increased to 25:1. However, the efficiency was significantly decreased at a molar ratio of 30:1 (Fig. 6). This result was also observed in the conjugation experiment of BSA.

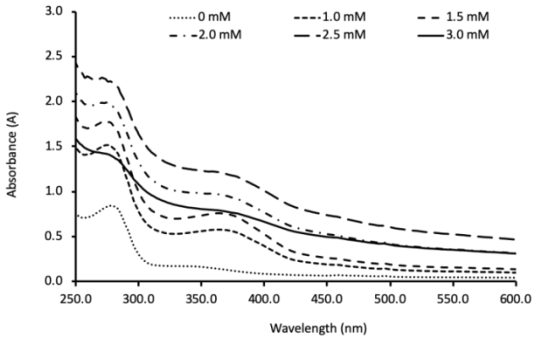

Figure 6. Spectral scanning results of KLH-TC conjugate solutions at different concentrations of tetracycline (1.0, 1.5, 2.0, 2.5 and 3.0 mM).

3.2.2. Determination of the formaldehyde concentration

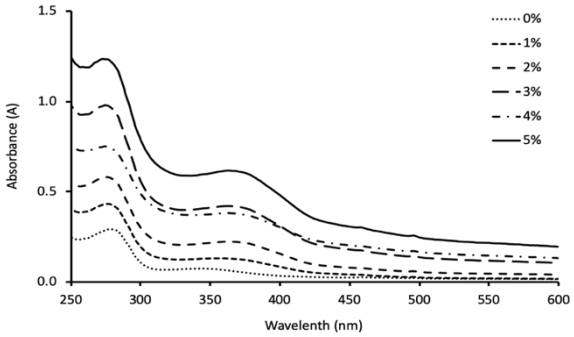

Figure 7. Spectral scanning results of KLH-TC conjugate solutions at different concentrations of formaldehyde (0, 1, 2, 3, 4 and 5 %).

Based on the above research results, a range concentration of formaldehyde (0 - 5 %) was utilized in the reaction. The results also showed that the absorption intensity increased with an increasing formaldehyde concentration in the reaction. Similarly, the maximum absorption wavelength at the first peak also tended to shift from the protein peak (278 nm) towards the formaldehyde peak (269 nm) (Fig. 7). These observations demonstrated that higher numbers of TC molecules were attached to KLH molecules with an increasing concentration of formaldehyde.

3.2.3. Effect of conjugation temperature and time

Similar to BSA-TC conjugation, the KLH-TC conjugation reaction was carried out at three different temperatures of 4 ^{o}C, 25 ^{o}C and 37 ^{o}C.The obtained results indicated that the suitable temperature for conjugation was 4 ^{o}C (Fig. 8a). In terms of reaction time, there was not much difference in conjugation efficiency for 4 - 6 hours. Therefore, the most suitable time to carry out the reaction was 4 hours (Fig. 8b).

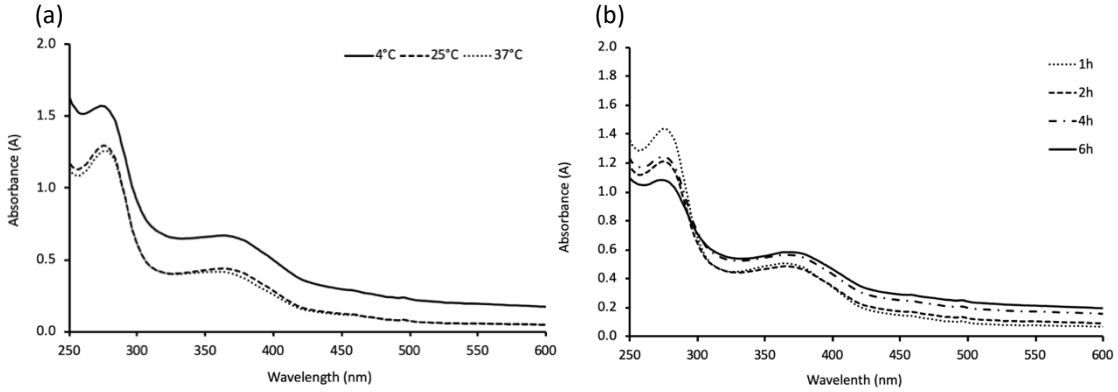

Figure 8. Spectral scanning results of KLH-TC conjugate solutions at different conjugation reaction temperatures (4 ^{o}C, 25 ^{o}C, and 37 ^{o}C) and with the different conjugation reaction time (1, 2, 4, 6 hours).

3.3. Production of polyclonal antibody against Tetracycline

The KLH-TC conjugates were injected into specific-pathogen free rabbitswith the addition of CFA in the first dose and IFA for the next doses [13]. The mycobacteria component in CFA attracts macrophages and other cells to the injection site, resulting in enhancing the immuneresponse [14]. Therefore, the CFA was used for the initial dose and the IFA was used for the subsequent boosts.The KLH-TC conjugate prepared in 1X PBS was mixed with Freund's adjuvant to produce a water-in-oil emulsion of immunogens. This emulsion helped to slowdown the release of LKH-TC antigen and, as a result, stimulated high and long-lasting antibody responses. The blood of rabbits was collected immediately before each infection to check antibodyproduction. Serum was obtained from each bleed.The antibody response of immunized rabbits was estimated by the ELISA method. Since the antisera as well as purified IgG antibodies may contain anti-KLH antibodies (due to using KLH-TC for immunization), the BSA-TC conjugate was used as a coating antigen to avoid cross-reaction. Figure 9 showed the dynamics of antibody responses during immunization of rabbits. The antibody titer increased from injection dose 1 to dose 6 and then decreased at doses 7 and 8. Whole blood of rabbits was collected 14 days after dose 7.

Since protein A is specific for the Fc region of IgG [15], therefore, the IgG antibody against tetracycline was purified using a Protein A affinity column. The protein patterns of whole serum, flow through fraction, and eluent fractions were analyzed by SDS-PAGE (Fig. 10a). The results showed the presence of two protein bands with a size of approximately 50 and 25 kDa (lane 4) in the eluent fraction corresponding to the IgG heavy and light chains, respectively. Observed results demonstrated that IgG was successfully purified from serum. The amount of purified IgG was determined as 1.11 mg/mL of serum. The relative purity of the IgG antibody solution was approximately 98 %.

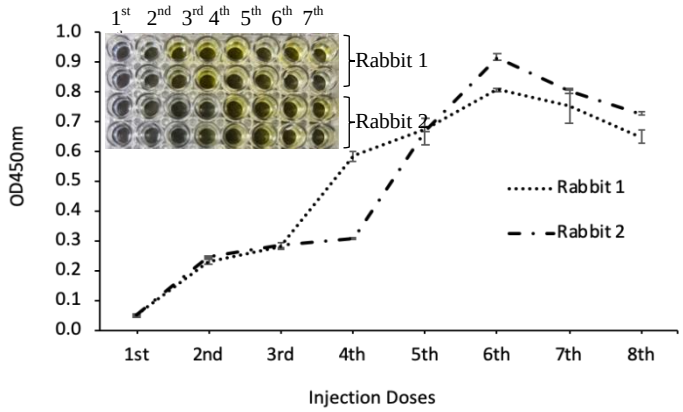

Figure 9. Immunological responsesof rabbits against tetracycline.

The biological activity of an anti-tetracycline polyclonal antibody was characterized by ELISA method. As mentioned above, the BSA and BSA-TC conjugate were coated on the bottom of the plate wells to avoid cross-reaction with KLH. Purified IgG was used as the primary antibody. The 1X PBS buffer was used as an alternative to the primary antibody in the control sample. The ELISA result showed that a significant signal was observed only when the ELISA well was coated with the BSA-TC conjugate (Fig. 10b). The obtained results demonstrated that the generated polyclonal antibody waste tetracycline specific. This antibody was quality enough in purity and specificity for several applications.

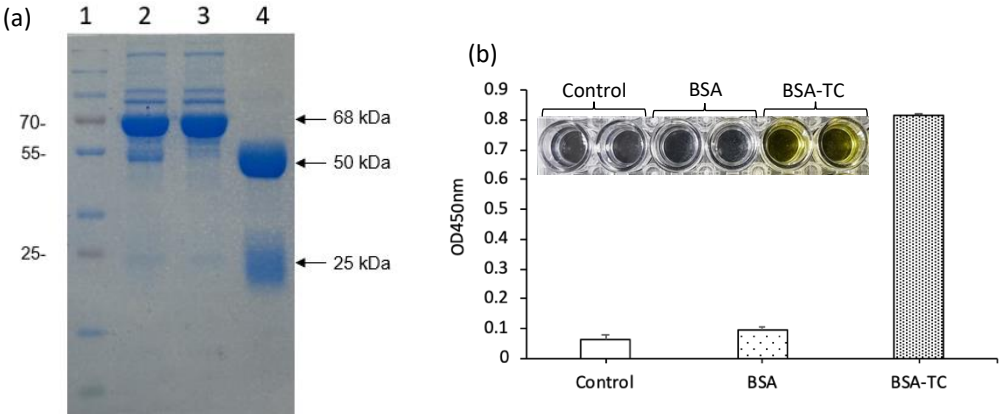

Figure 10. Purification of polyclonal antibody from KLH-TC - immunized rabbit serum and biological activity of purified antibody. Lane 1, protein marker; lane 2,whole serum; lane 3, flow through; lane 4, eluted IgG fraction.

3.4. Preliminary application of prepared anti-tetracycline polyclonal antibody for generating the lateral flow immunoassaytest strip

The lateral flow immunoassay (LFA) test strip has been a well-established diagnostic tool. The test strip is a rapid analytical instrument that works on the principle of immunochromatography. Two commonly used LFA test strip formats are direct LFA and competitive LFA. The competitive LFA method has been developed for the determination of small molecules such as mycotoxins and antibiotics. In which the reaction and signal visualization take place in the nitrocellulose membrane. Since TC was a small molecule, the LFA was designed as a competitive immunoassay. In this study, the generated anti-tetracycline polyclonal antibody was utilized to conjugate with gold nanoparticles to generate the conjugate pad and was included in the LFA strip. The BSA-TC conjugate and protein A were immobilized at the T-line and C-line, respectively. Figure 11 showed the appearance of two significant bands (T-line and C-line) in the case of negative sample and only a single band (C-line) in the case of a positive sample. The result indicated that the generated polyclonal antibody included in the conjugate pad of the LFA strip recognized and captured tetracycline in the tetracycline – BSA conjugate immobilized at the T-line position to generate the signal. In the case of a positive sample (the presence of TC in the sample), the colloidal gold-labelled anti-TC antibody in the conjugate pad would bind to the TC when it moved through the conjugate pad of the strip and thus not be available for TC-BSA at the T-line position, resulting in no signal being formed at the T-line position. The obtained results revealed that the generated anti-TC polyclonal antibody could be used for the development of the lateral flow immunoassay test strip to detect tetracycline in a sample. The specificity and other characteristics of the test strip will be determined in further study.

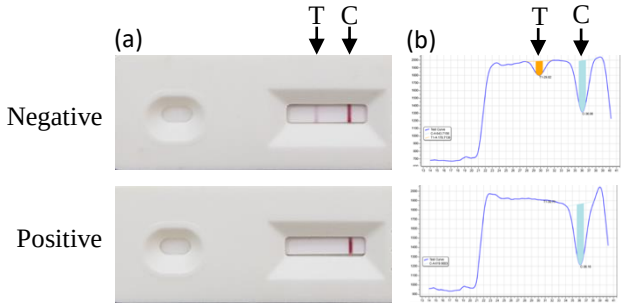

Figure 11. Lateral flow immunoassay for detection of Tetracycline. (a) rapid test strips and (b) signal band intensities of negative and positive samples.

4. CONCLUSIONS

The present study has successfully established the suitable conditions for the conjugation of tetracycline to carrier proteins such as BSA and KLH. The KLH-TC conjugate was highly immunogenic in rabbits. The generated polyclonal antibody could recognize tetracycline and could be used for the development of lateral flow immunoassay test strips for the detection of tetracycline and other applications.

Credit authorship contribution statement. Ngo Thu Huong: Conceptualization, methodology, investigation, formal analysis, preparation of the draft, revision of the article; Hoang Thi Huyen: Investigation, formal analysis

Truong Quoc Phong: Conceptualization, methodology, investigation, formal analysis, preparation of the draft, revision of the article and final approval of the verion to be published.

Declaration of competing interest. The authors declare that they have no conflicts of interest in relation to this article.

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