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Contributed Paper

Establishment of Competitive ELISA for Detection of Chloramphenicol

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ABSTRACT

Chloramphenicol adulterated in animal supplies is one of essential global concerns. In this study, the competitive ELISA for measuring the chloramphenicol level was established. The immunogen, chloramphenicol conjugated bovine serum albumin, was constructed with a modified chemical cross-linking procedure. A rabbit was injected for hyperimmune serum production and the presence of anti-chloramphenicol antibodies was demonstrated by indirect ELISA and Western blotting. The suitable dilution of serum containing anti-chloramphenicol antibodies was directly used in the assay. The interference of anti-bovine serum albumin antibodies appeared in the hyperimmune serum was prevented by coating the ELISA well with ovalbumin-chloramphenicol. In addition, the antibody populations, which reacted with bovine serum albumin, were neutralized after diluting in buffer containing bovine serum albumin. The working range of the assay was 10-1,280 ng/ml.

Keywords: chemical conjugation, chloramphenicol, competitive ELISA, hapten, polyclonal antibodies.

1. INTRODUCTION

Chloramphenicol (CAP) is an effective antibiotic agent against a wide range of bacteria, including gram-positive and gram-negative cocci and bacilli [1-3]. However, due to its serious side effects, such as bone marrow depression, aplastic anemia and other serious blood disorders, CAP was prohibited from its use in food-producing animals [4-5]. A zero tolerance level for CAP residues in animal products has been established worldwide by the Food and Drug Administration. In Thailand, CAP level in food products must not exceed the maximum residue level (MRL) at 0.3 μg/kg. Regardless of the above regulations, illegal use of CAP still remains due to its potency, availability and relatively inexpensive [4]. In response to the recent legislation in many countries, methods that can carry out large-scale monitoring programs of this substance are therefore highly desirable.

Currently, a variety of methods have been introduced for monitoring CAP in food products and biological samples [6-11]. These include chromatographic [6-8], microbiological [9], enzymatic [10] and immunological assays [11]. Chromatographic techniques such as GC and HPLC offer great sensitivity of the detection. However, the techniques often laborious, require highly specialized technicians and expensive instruments. Moreover, only one sample can be handled at a time. An alternative for the limitations inherent to these techniques is the detection of CAP residues by enzyme-

linked immunosorbent assays (ELISAs) [12]. The methods are not only highly specific and sensitive, but also quick and easy to handle. These allow collections of samples to be analyzed simultaneously. Hence, ELISAs are suitable analytical tools when a high number of samples need to be analyzed.

In this article, we describe the preparation and characterization of polyclonal antibodies to CAP and the development of sensitive Competitive Enzyme-linked Immunoassay (CELIA) system for detection of CAP.

2. MATERIALS AND METHODS

2.1 Chemicals and Instruments

All chemicals were purchased from commercial suppliers and used as received. Succinic anhydride, 4-(dimethylamino)-pyridine (DMAP), pyridine and *N*-hydroxy succinimide (NHS) were purchased from Fluka, USA. Diisopropyl carbodiimide (DIC), chloramphenicol (CAP), chloroform-d ($CDCl_3$) and Bovine serum albumin (BSA) were obtained from Sigma-Aldrich, USA. Ovalbumin (OVA) was obtained from Calbiochem, USA. Conjugated CAP-bovine serum albumin (cCAP-BSA) was purchased from Biotrend, USA. NMR spectra were recorded on Bruker *AVANCE* N M R spectrometer (400 MHz for ^1H). Chloro-form-d ($CDCl_3$) was used as the solvent. Chemical shift values (δ) are reported in ppm relative to internal tetramethylsilane. Coupling constant (J) are expressed in Hz and the abbreviation s, d, m, and br represent-splitting pattern as singlet, doublet, multiplet and broad NMR peak, respectively. Optical density in ELISA was measured with a microplate reader (Tecan, Austria).

2.2 Synthesis of CAP Succinate

A previously reported procedure was modified for the preparation of CAP succinate [13]. In brief, CAP (2.00 g, 6.19 mmol) was added into a stirred mixture of succinic anhydride (0.72 g, 7.19 mmol) and DMAP (0.037 g, 5 mol %) in 10 ml pyridine. After sonicating for 10 min, the mixture was allowed to stir at ambient temperature for 18 h. Pyridine was then removed under reduced pressure, and the crude residue was re-dissolved in 10 ml ethyl acetate. After washing and drying (Na_2SO_4), the solvent was removed and the crude residue was purified via flash chromatography using 50-70% ethyl acetate /hexane as eluent. The desired product was obtained in 72 % yield (1.88 g) as colorless oil which solidified upon standing. R_f 0.3 (80 % ethyl acetate/hexane); ^1H NMR ($CDCl_3$) δ 8.08 (d, J = 8 Hz, 2H), 7.60 (d, J = 8 Hz, 2H), 7.45 (s, 1H), 6.01 (s, 1H), 5.10 (br, 1H), 4.35 (br, 2H), 4.13 (br, 1H), 2.94 (m, 2H), 2.82 (m, 2H).

2.3 Synthesis of NHS Ester of CAP Succinate

NHS (0.139 g, 1.21 mmol), DIC (0.166 g, 1.315 mmol) and DMAP (0.006 g, 0.005 mmol) were added into a stirred solution of CAP succinate (0.42 g, 1.0 mmol) in dichloro-methane/ DMF 4:1 (5 ml) at 0 °C. The mixture was stirred at room temperature for 3 hours. After filtered and evaporated, the residue was re-dissolved with a small amount of dichloromethane. The crude mixture was purified via flash chromatography with 50-60% ethylacetate/hexane to afford 0.45 g (85 %) of product as colorless oil. This material was freshly prepared prior to the conjugation step to avoid decomposition of the active ester.

2.4 Synthesis of CAP-Protein Conjugates

The hapten was covalently attached to carrier proteins using active ester method [14]. The proteins used were BSA and OVA. The detail procedures were as follows. A solution of NHS ester of CAP-succinate in 1.00 ml of DMF (calculated to be a 40-fold molar excess over the protein) was added dropwise for over 30 minutes into a stirred solution of protein (100 mg) in phosphate buffer (0.05 M, pH 8.5, 7.50 ml) and DMF (1.00 ml) at 0 °C. The stirring was continued for 1 h at room temperature and 72 h at 4-5 °C. The pH of this solution was kept at 8.5 via addition of

sodium bicarbonate. The resulting solution was centrifuged at 8,000 g at 4 °C for 30 minutes and dialysis in water/ammo-nium hydroxide buffer (pH 8.0) at 25 °C for 8 h and at 4 °C for 16 h with two dialysate changes. The resulting solution was lyophilized to give the white protein conjugate.

2.5 Verification of Conjugate by Indirect ELISA

The commercial conjugated CAP-bovine serum albumin (cCAP-BSA) (Biotrend, USA) was used as a standard for evaluating the presence of CAP epitope in the in-house synthesized CAP-bovine serum albumin (CAP-BSA) and CAP-ovalbumin (CAP-OVA). ELISA wells were separately coated overnight with 50 µl of 10 µg/ml of cCAP-BSA, CAP-BSA or CAP-OVA. After washing step, wells were blocked with 2% BSA/PBS for 2 h. Subsequently, 50 µl of 1:1,000 dilution of sheep anti-CAP antibodies purchasing from Biotrend in 2% BSA/PBS was added into the washed wells. The bound sheep anti-chloramphenicol antibodies were monitored by 1:3000 dilution of HRP conjugated rabbit anti-sheep immunoglobulins antibody. 100 µl of TMB/H_2O_2 substrate-buffered solution was then applied after 1hour incubation and washing step. The equal amount of 1 N HCl was added to each well when 15 min substrate incubation was reached. The enzymatic reaction was measured spectro-photometrically at the wavelength of 450 nm.

2.6 Antiserum Production

CAP antiserum was prepared according to the following schedule. An albino New Zealand rabbit was immunized with 0.25 mg of CAP-BSA antigen in 1.0 ml of a 1:1 mixture of complete Freund's adjuvant by subcutaneous multi-site injections. Booster injections of 0.25 mg of antigen in 1.0 ml of a 1:1 mixture of PBS were given one week post- immunization, followed by three additional boosters given at couple-week intervals. The blood was collected every time before each immunization for monitoring the antibody response. The blood was allowed to clot at 37°C for 2 h, and the serum was collected by centrifugation. The hyperim-mune serum obtained after 2 weeks of the last immunization was used through out the study.

2.7 Analysis of Rabbit Anti-CAP Antibodies by Indirect ELISA

The Rabbit anti-CAP antibodies were determined by indirect ELISA. In brief, ELISA wells were coated overnight with 50 µl of 5 µg/ml CAP-OVA (or CAP-BSA) in carbonate buffer. Excess antigens were removed by 3-time washing with washing buffer (0.05% Tween 20 in PBS). The coated wells were subsequently blocked with 200 µl of 2% BSA/PBS for 1 h after extensive washes, 50 µl of the serum (1:8000 in 2%BSA/PBS) was applied to the coated wells. After washing, 50 µl of HRP-swine anti-rabbit immunoglobulins conjugate (1:3000 dilution) was applied to each well and incubated for another 1 h. Finally, 100 µl of TMB/H_2O_2 substrate-buffered solution was added to the washed wells and let stand for 15 minutes before adding 100 µl of 1 N HCl. The enzymatic reaction was determined at the wavelength of 450 nm.

The above procedure was also used for evaluation of rabbit anti-CAP antibodies specificity. In this study, ELISA wells were instead coated with 50 µl of 5 µg/ml CAP-OVA and OVA, and 1:1000 in 2%BSA/PBS of serum was applied to the coated wells.

2.8 SDS-PAGE and Western Immunoblotting

SDS-PAGE was performed according to the technique described by Laemmli [15]. Reducing forms of CAP-BSA and unconju-gated BSA were electrophoretically separated in 12% polyacrylamide separating gel. Sample preparation was performed in the presence of 2-mercaptoethanol and SDS under heat denaturation. Western blotting was performed to transfer the separated polypeptides to the PVDF membrane as described by Tawbin

[16]. The membrane was blocked with 5% BSA/PBS. Subsequently, the CAP-BSA immunized rabbit serum (1:2,500 in skim milk/PBS and 1:2,500 in BSA/PBS) was applied to the blocked membrane. The excess antibody was washed out and HRP-swine anti-rabbit immunoglobulins conjugate was added. The TMB/H_2O_2 immunoblotting substrate-buffered solution was used to localize bound antibodies on the reaction membrane. The chromogenic product was observed after 15-20 min incubation. The molecular size of each reactive band was calculated in relative to the standard proteins.

2.9 Determination of Rabbit Anti-chloramphenicol Titer

Titers were determined for the antiserum, which was assessed for antibiotic-binding activity. ELISA was performed as previously described in 2.6 except coating ELISA well with only CAP-OVA and 2-fold serial dilutions of individual rabbit sera performed in 2% BSA/PBS were added to assayed wells.

2.10 Establishment of CELIA

An indirect CELIA was implemented according to the following process. 50 µl of CAP-OVA antigen at a concentration of 5 µg/ml in carbonate buffer was added to polystyrene wells (Nunc, Sweden) and allowed to passively adsorb for 16 h at 4°C. After washing the wells once with washing buffer, 200 µl of 2% BSA in PBS (PBS-BSA) was added to each well and incubated for 2 h at RT. After removal of the PBS-BSA blocking solution and washing three times with washing buffer, 25-µl of various CAP standard concentrations or test samples was added to respectively designated wells. Then, 25 µl of suitable dilution of CAP antiserum in PBS was added, mixed, and incubated for 1 h at RT. Wells were washed three times with washing buffer and then 50 µl of HRP-swine anti-rabbit immunoglobulins at dilution of 1:2,500 was added, mixed, and incubated for 1 h at RT. The wells were then washed three times with washing buffer, and 100 µl of a TMB/H_2O_2 substrate-buffered solution was added, mixed, and incubated for 15 min at RT. The enzymatic reaction was stopped by the addition of 100 µl of 1 N HCl solution. The absorbance values were measured at 450 nm in a spectrophotometer.

The values of optical density were converted to percent inhibition (B/B_0) according to the formula: $\%B/B_0 = B/B_0 \times 100$, where B_0 = absorbance in well containing no CAP competitor, B = absorbance in well containing a particular CAP concentration.

3. Results and Discussion

3.1 Synthesis of Haptens and Conjugates

CAP is a hapten, incapable of eliciting antibody response by itself; thus the production of CAP-specific antibodies presents an obvious challenge. Two types of CAP derivatives have been prepared and used to conjugate with proteins. One derivative was obtained by reducing the nitro group of CAP and coupled to BSA by diazotization [17]. Another form of CAP derivative was prepared by linking succinyl moiety to the terminal hydroxyl group of CAP. This material was linked to proteins by the classical carbodiimide reaction [18].

Previous studies have shown that both the dichloroacetamido and nitrophenyl groups of CAP are necessary for the immunogenic reactivity of this hapten [19, 20]. In this study, CAP derivative containing a succinate bridge was therefore synthesized and used to prepare two types of protein conjugates, CAP-BSA and CAP-OVA (Figure 1). By using modified procedures of the previously described literature [13], CAP succinate was obtained in 72 % yield. The NMR data confirm the presence of the desired product. After activation of CAP succinate with NHS, the active ester of hapten was conjugated to two carrier proteins; BSA and OVA.

The presence of CAP on the conjugated proteins was validated by indirect ELISA using commercialized anti-chloramphenicol antibodies (Table 1). The absorbance unit

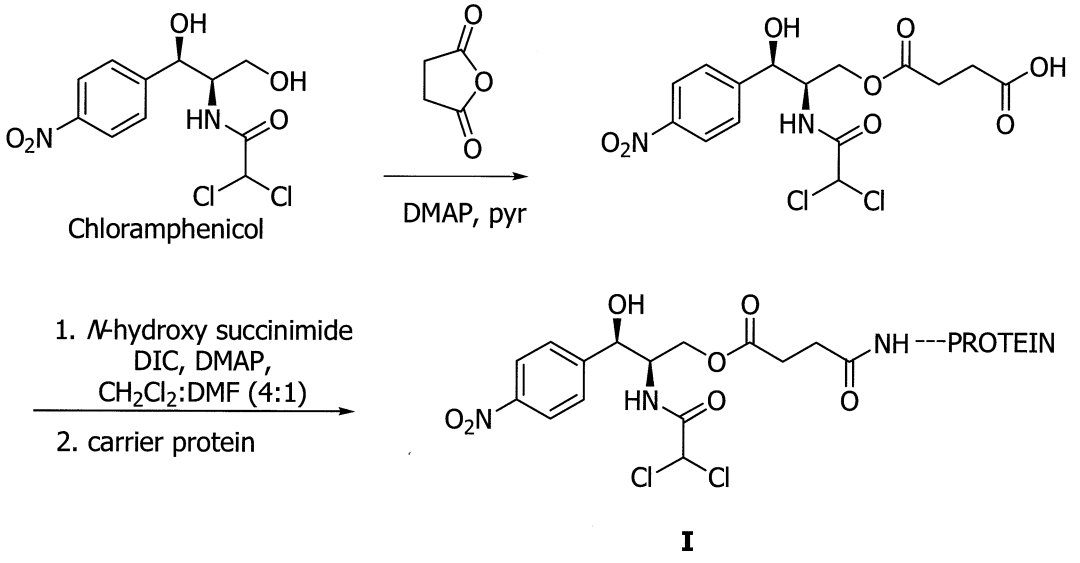

Figure 1. Synthesis of CAP conjugated proteins.

obtained from CAP-BSA was greater than that of the commercialized CAP-BSA suggesting that our CAP-BSA conjugate contained higher amounts of CAP molecule. The CAP-OVA product also gave a positive signal in comparison with the control well, but not as strong as CAP-BSA. This could be resulted from more lysine residues per mole in BSA than in OVA. Consequently, the prepared CAP-BSA was used as immunogen for production of anti-CAP hyperimmune serum.

3.2 Comparison of Antibody Activity Against CAP-BSA and CAP-OVA

The presence of anti-chloramphenicol antibodies in the immunized rabbit serum was clearly demonstrated by indirect ELISA using two different antigens i.e. CAP-BSA and CAP-OVA. (Table 2) The absorbance in well coated with CAP-BSA was significantly higher than in CAP-OVA coated well. The conjugate control well gave very low absorbance unit which implied the specificity of assay.

Table 1. Indirect ELISA for validation of prepared chloramphenicol conjugated BSA and OVA.

Coated Antigen 10 μg/ml	Sheep anti-chloramphenicol antibodies
	Absorbance unit (λ_{max} 450 nm)
cCAP-BSA	0.706
CAP-BSA	1.313
CAP-OVA	0.489
No coat	0.106

*Commercialized chloramphenicol conjugated BSA from Biotrend, USA.

Table 2. Detection of anti-chloramphenicol antibodies in hyperimmune serum by using two different coated antigens.

Sample	Absorbance unit (λ_{max} 450 nm)	
	CAP-OVA	CAP-BSA
Rabbit serum (1:8,000 in 2%BSA/PBS)	0.779	1.594
No serum	0.068	0.066

3.3 Specificity of CAP-BSA Immunized Rabbit Serum

Indirect ELISA was used to evaluate the specificity of anti-chloramphenicol antibodies. As shown in Table 3, when the plate was applied with 1:1000 dilution of serum, CAP-OVA coated well gave the highest absorbance unit, whereas no signal could be observed in the OVA coated and uncoated wells. The data suggested that the rabbit anti-chloramphenicol antibodies specifically reacted with CAP determinant without cross-reaction with OVA.

Table 3. Determination of specificity of anti-chloramphenicol antibodies by indirect ELISA.

Rabbit serum dilution	Absorbance unit (λ_{max} 450 nm)		
	Coated antigen		
	CAP-OVA	OVA	No coat
1:1,000	1.431	0.117	0.062
No serum control	0.062	ND	ND

3.4 Demonstration of Anti-chloramphenicol Antibodies by SDS-PAGE and Western Immunoblotting

Figure 2 showed Western immunoblotting analysis of the obtained CAP-BSA. CAP-BSA conjugate was loaded in lane 1, 3, 5, 7, and unconjugated BSA, used as a control, was applied in lane 2, 4, 6. The membrane was probed with an appropriate dilution of CAP-BSA immunized rabbit serum. According to Figure 2 (set A), lanes 1 and 2 showed positive bands in reacting with serum diluted in skim milk. In comparison to the positive control, probed with commercial sheep anti-CAP antibodies (set C), and the negative control (without serum, lane 7), the specific band was observed only on lane 3 but not in lane 4 (set B). Since the serum was diluted in BSA/PBS, the data suggested anti-BSA antibodies blended in serum were successfully blocked, confirming the existence of anti-CAP antibodies in the serum.

3.5 Determination of Rabbit Anti-chloramphenicol Titer

The titer of anti-chloramphenicol polyclonal antibody in immunized rabbit serum was determined for its titer by indirect ELISA. The 2-fold serial dilution of immunized rabbit serum starting from 1:2,000 to 1:128,000 was made and added to the CAP-OVA coated wells. Subsequently, HRP-swine anti-rabbit immunoglobulins conjugate was added to each reaction well. The absorbance value of individual well was measured and plotted as shown in Figure 3. When the O.D. of 0.5 was used as a cut of, the approximate serum titer was 1:16,000.

3.6 Establishment of CELIA

Regarding to the titration curve of rabbit anti-chloramphenicol antibodies obtained, we compared two selected dilutions (1:16,000 and 1:20,000) for setting up a standard curve in competitive ELISA. Various concentrations of standard chloramphenicol were added for competing for the anti-chloramphenicol

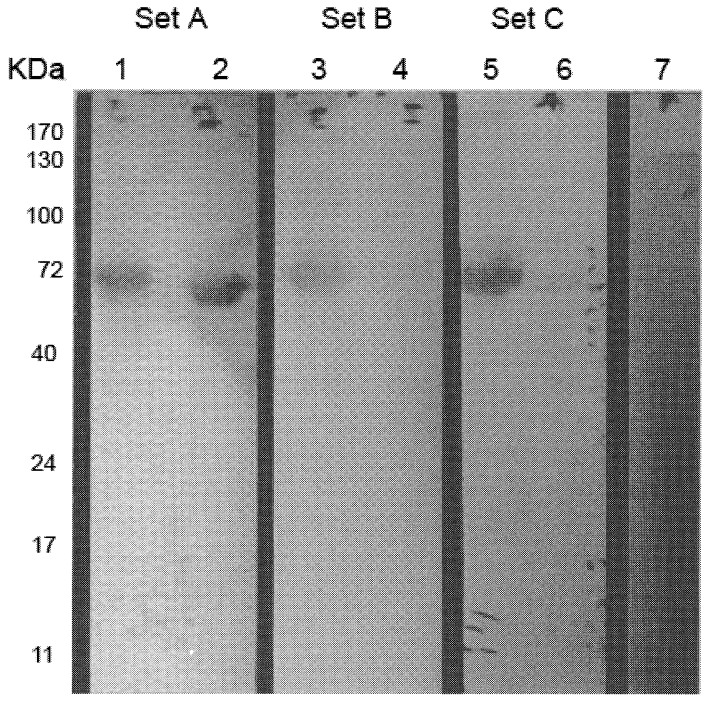

Figure 2. Western immunoblotting analysis showing the specific immunological reaction of rabbit anti-chloranphenicol. CAP-BSA conjugate was loaded into lane 1,3,5, and 7, whereas BSA was loaded in to lane 2,4 and 6. Set A was reacted with rabbit serum diluted in skim milk PBS. Set B was probed with rabbit serum diluted in BSA PBS. A commercial sheep anti-CAP antibody was applied into set C. Lane 7 was subjected as negative control without rabbit serum added.

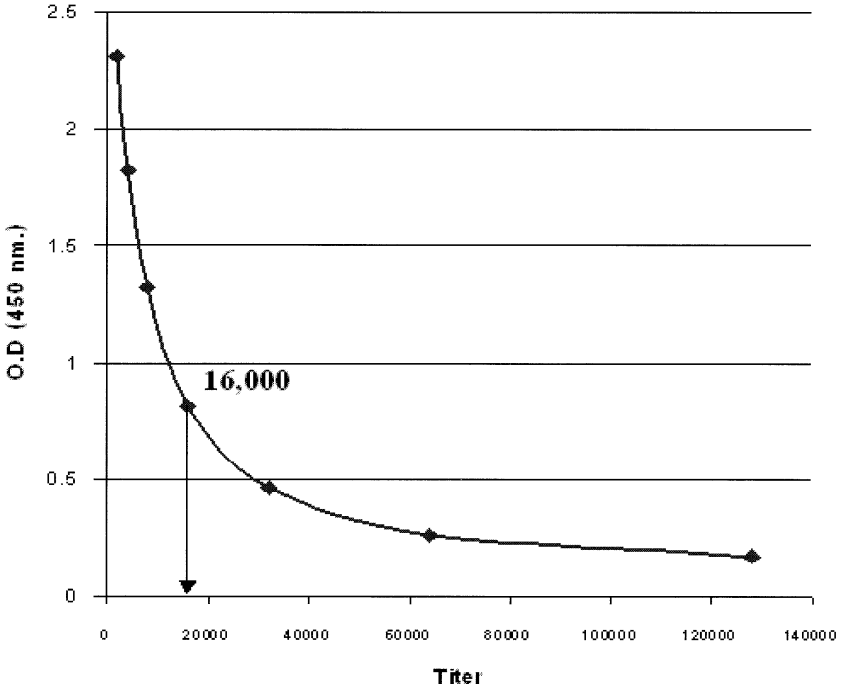

Figure 3. Titration curve of CAP-BSA immunized rabbit serum by indirect ELISA.

antibodies with the coated CAP-OVA. The CAP-OVA was used instead of CAP-BSA to prevent the anti-BSA or antibody against the junction epitope of BSA and CAP that might occur in CAP-BSA immunized rabbit serum. The anti-BSA was shown when an appropriate dilution of CAP-BSA immunized rabbit serum in blocking solution containing skim milk was applied to the BSA coated well. Whereas, no signal could be observed in case that blocking containing BSA was used. These antibodies, if present, will not be blocked with free chloramphenicol. The sensitivity of this test will, of course, be reduced. With this strategy, monoclonal antibodies against chloramphenicol are not required.

Comparing between two sets of standard curve obtained, the final dilution of 1:16,000 was suggested for this lot of hyperimmune serum. The linearity of standard curve was more applicable at this dilution (Figure 4). The detection range was 10-1,280 ng/ml which should be suitable for screening the contamination or misused of chloramphenicol in samples.

Recently, Impens *et al.* [21]. developed the competitive ELISA using purified rabbit anti-CAP for coating, and CAP-horseradish peroxidase as a probe. The detection range was 0.25-2.0 mg/kg or ng/ml. Although the sensitivity was higher than that of our assay, the detection range was narrower. Thus, for high level contamination of CAP in samples, our system should be more suitable since no further dilution is required.

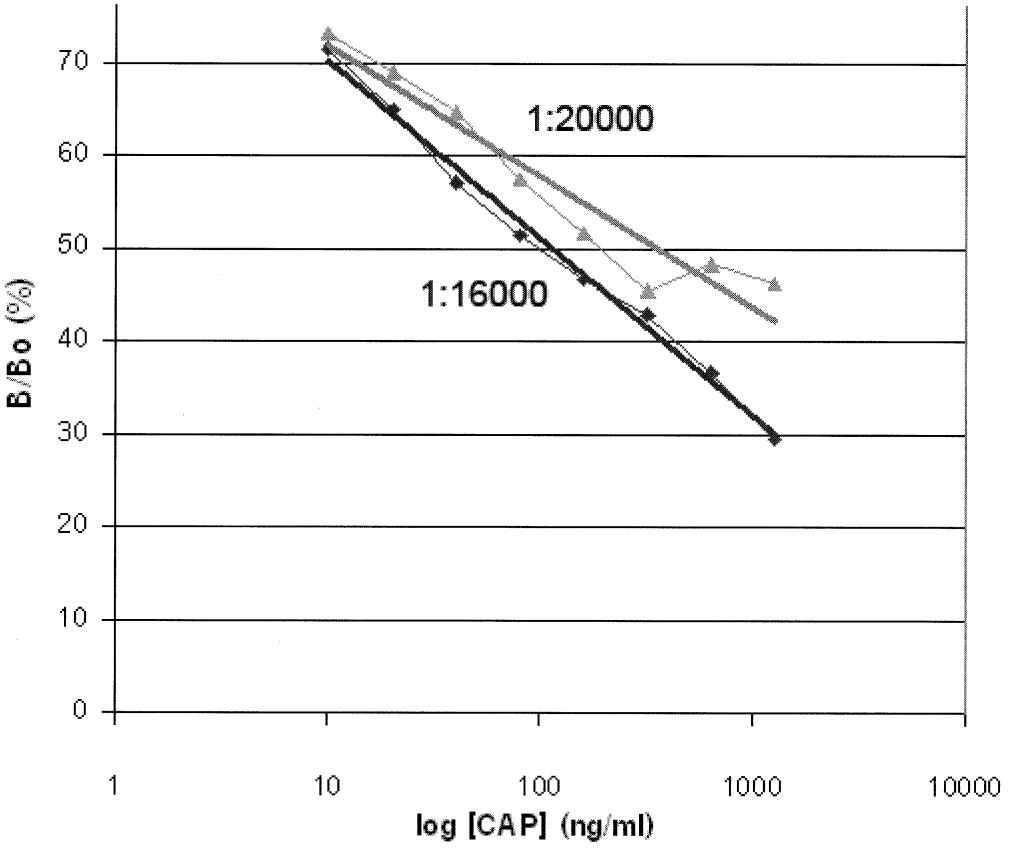

Figure 4. Comparison of standard curve in the established CELIA. Thin-line designed the raw data of calculation. The average value of each dot was demonstrated as trend-line (thick-line).

4. CONCLUSIONS

In this study, we successfully established the CELIA for the detection of chloramphenicol. Anti-CAP hyperimmune serum can directly be used for simplifying the development of in-house immunoassay. This can greatly reduce the cost of production of a drug specific monoclonal antibody. Moreover, enzyme conjugated drug is not required in detecting the antigen-antibody interactions. This should give an additional advantage in avoiding complicated hapten synthesis of structurally complex molecules. The sensitivity of the developed CELIA was obtained in a nanogram level. Its broad linearity range of the detection should be suitable for screening the contamination of chloramphenicol in samples. The established method will be evaluated for its prediction value, precision and accuracy in CAP measurement in animal supplies.

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