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Improved group determination of tetracycline antibiotics in competitive enzyme-linked immunosorbent assay

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Seven tetracycline (TC) antibiotics were used as haptens for synthesis of conjugated antigens and as standards in indirect competitive enzyme-linked immunosorbent assay (ELISA). Seven conjugates were prepared by formaldehyde condensation (f) and another seven antigens were synthesised by linking TCs with periodate-oxidised transferrin (TF(pi)). Two gelatine–lymecycline (LC) conjugates were the products of glutaraldehyde and activated esters reactions. To estimate the influence of solid-phase antigen on assay specificity, all the conjugates were absorbed on polystyrene plates and using anti-bovine serum albumin–TC(f) sera, the ELISAs were developed and compared. The variant with immobilised TF–chlortetracycline (CTC(pi)) showed the best group specificity: recognition of seven TCs differed only in 5.3 times. This assay displayed sensitivity 0.1 ng/ml and cross-reactivity indexes: TC (100%), CTC (105%), oxytetracycline (19.8%), methacycline (23.6%), minocycline (70%), doxycycline (25%) and LC (50%). The dependence of ELISA specificity on immobilised antigen spatial structure was demonstrated. Solid-phase conjugate selection is a useful tool for modifying assay specificity.

Keywords: tetracyclines; immunoassay; group specificity; immobilised antigens

Introduction

Heretofore, a purposeful developing group-specific method for determination of tetracycline (TC) antibiotics using immunoassay unlike to sulphonamides, aminoglycosides, fluoroquinolones and penicillins has not been a subject of publications yet (Burkin, 2008; Korpimäki et al., 2004; Loomans, van Wiltenburg, Koets, & van Amerongen, 2003; Usleber, Litz, & Martlbauer, 1998). A few of these substances utilised in veterinary practice – TC, 7-chlortetracycline (CTC), oxytetracycline (OTC) and doxycycline (DC; Council Regulation (ECC) N 2377/90) and minimal distinctions in their chemical structures would seem to present no problems for developing of group-specific assay. However, literature data showed that recognition of these substances by antibodies with equal or near efficacy proved to be insufficient. Thus, several works have been noted CTC revealed 10 and 14% of TC activity (Jeon, Kim, Paeng, Park, & Paeng, 2008; Pastor-Navarro, Moraisa, Maquieira, & Puchades, 2007), the same indexes for OTC varied between 0 and 30% and cross-reactivity of DC was not more than 5% level (Faraj & Ali, 1981; Jeon et al., 2008; Kumar, Thompson,

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Singh, Chander, & Gupta, 2004; Pastor-Navarro et al., 2007; Zhang, Lu, Liu, Zhao, & Xi, 2007; R-Biofarm AG).

This work presents a comparison study of reactions between antibodies against TC and immobilised conjugated antigens of different structural design and its role for developing enzyme-linked immunosorbent assay (ELISA) to TCs with maximum prominent group specificity.

Materials and methods

Chemicals and reagents

Hydrochlorides of TC, CTC, OTC, DC, methacycline (MeC), human transferrin (TF), N-hydroxysuccinimide, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) and *o*-phenylenediamine were purchased from Fluka-Sigma-Aldrich (Moscow, Russia). Lymecycline (LC) was from Galderma SA (La Defense, France), minocycline hydrochloride (MiC) and glutaraldehyde were from Serva (Heidelberg, Germany). Peroxidase-conjugated donkey antibodies to rabbit IgG, bovine serum albumin (BSA) and gelatine (Gel) were obtained from Pharmatch (Moscow, Russia). Antiserum against BSA-TC(f) conjugate prepared by formaldehyde condensation method was kindly provided by Burkin AA and Kononenko GP (Department of Mycotoxicology, All-Russian Research Institute of Veterinary Sanitation, Hygiene and Ecology).

Preparation of standards

To prepare standards, the doses of weighed substances were poured with estimated volumes of distilled water or dimethylformamide (DMF). Prepared solutions were diluted by 100 times with water and analysed spectrophotometrically using Hitachi-557 device (Japan). The concentration of solutions was determined by the optical density at maximum absorption ranged from 243 to 270 nm using A1%/1 cm coefficients cited for these substances (Clark, 1986).

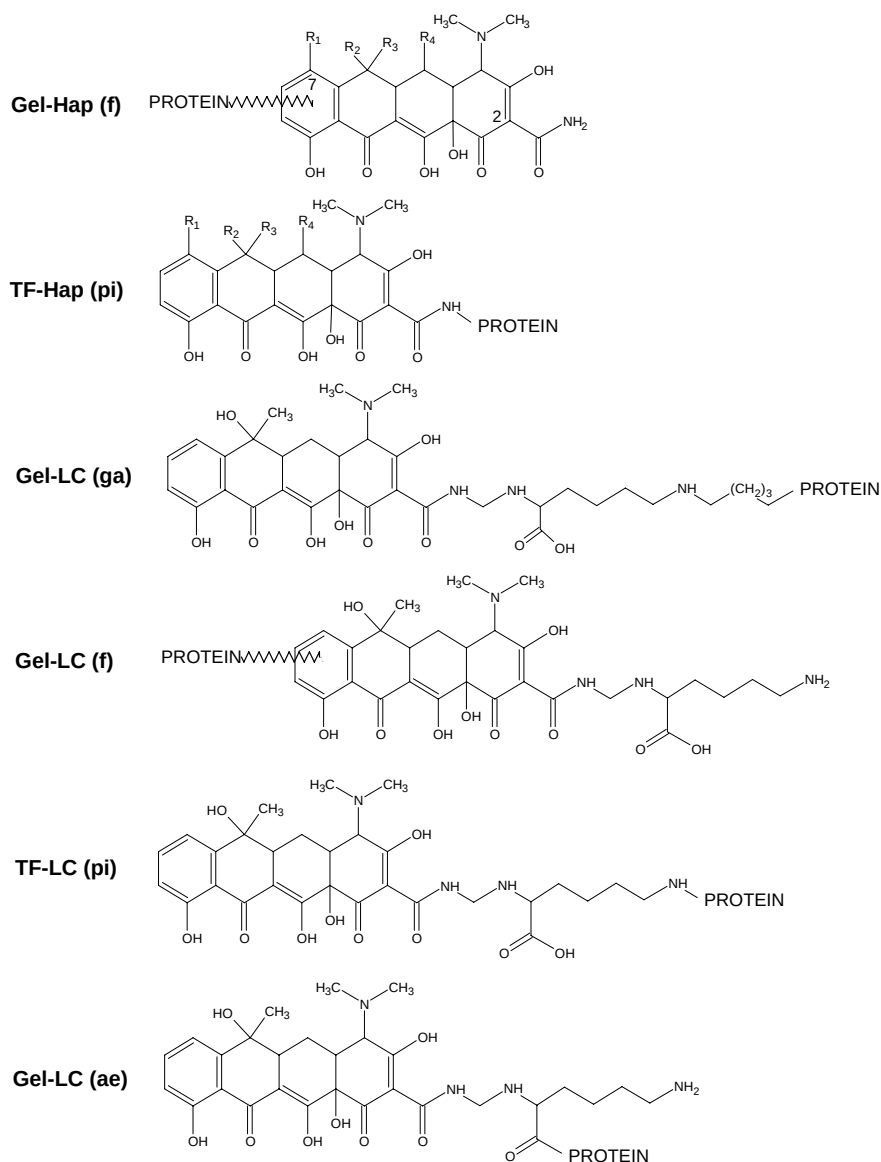
Conjugated antigens synthesis

Gel-TC(f), *Gel-LC(f)*, *Gel-CTC(f)*, *Gel-OTC(f)*, *Gel-MeC(f)*, *Gel-MiC(f)*,
Gel-DC(f)

This panel of conjugated antigens was prepared in the similar manner as immunogen. Seven mixtures of Gel (4 mg, 0.025 μ mol) in 1 ml, each representative of TC group (TC, LC, CTC, OTC, MeC, MiC and DC) taken in 100-fold molar excess (2.5 μ mol) in 0.1–0.4 ml of DMF and 0.3 ml 37% formaldehyde solution (3690 μ mol) were stirred at 30°C for 16 h and then dialysed three times against 1000 volumes of 0.5% NaCl.

TF-TC(pi), *TF-LC(pi)*, *TF-CTC(pi)*, *TF-OTC(pi)*, *TF-MeC(pi)*, *TF-MiC(pi)*,
TF-DC(pi)

Crystal sodium periodate (21 mg) was added to 5 ml solution of human TF (28 mg) and mixed for 15 min with magnet stirrer. Oxidised glycoprotein was dialysed against 5 l of 0.01M acetate buffer (pH 5.0) during the night at 4°C. The volume of resulted



Hapten (Hap)	R ₁	R ₂	R ₃	R ₄
TC	- H	- OH	- CH ₃	- H
CTC	- Cl	- OH	- CH ₃	- H
OTC	- H	- OH	- CH ₃	- OH
MeC	- H	= CH ₂		- OH
MiC	- N(CH ₃) ₂	- H	- H	- H
DC	- H	- H	- CH ₃	- OH

Figure 1. Chemical structures of solid-phase conjugated antigens synthesised on the base of tetracycline haptens.

dialyzate was divided into seven equal portions and added to solutions of antibiotics in 100-fold molar excesses. These mixtures were stirred for 2 hours and pH was adjusted to 8–9 using 3M NaOH. Then 0.1 ml of sodium borohydride solution (1 mg/ml) was added and mixed for another 2 hours. The resulted conjugates were dialysed exhaustively against 0.15M phosphate buffered saline, pH 7.0 (PBS).

Gel-LC(ga)

To 1 ml solution containing Gel (4 mg, 0.025 μ mol) in carbonate–bicarbonate buffer (CBB), pH 9.5 100-fold molar excess of LC (1.6 mg, 2.5 μ mol) in 0.4 ml was added together with 30 μ l freshly prepared 2.5% solution of glutaraldehyde. Followed by 2 h stirring at room temperature, each reaction mixture was supplemented with 0.1 ml of aqueous solution of sodium borohydride (1 mg/ml), maintained in a refrigerator for 1 h with periodic stirring and dialysed against 2×5 l of PBS for two days.

Gel-LC $\times 10(ae)$, Gel-LC $\times 40(ae)$

The 10 mg/ml solutions of N-hydroxysuccinimide and EDC (29 μ l and 48 μ l, respectively, 2.5 μ mol) were added to LC (753 μ g, 1.25 μ mol) in 0.5 ml DMF. After 2 h stirring at room temperature, 0.1 and 0.4 ml of reaction mixture was added dropwise to Gel solutions (4 mg, 0.025 μ mol) in 1 ml of CBB, incubated during 16 h at room temperature and dialysed against 0.5% NaCl.

All the dialyzates were supplemented with equal volumes of glycerol and stored at -10°C as solutions with concentration of 1 mg/ml. The linkage formation between hapten and carrier was proved by registration of specific changes in UV spectrum of conjugate in comparison with carrier spectrum and/or by interaction with antibodies in ELISA.

Enzyme-linked immunosorbent assays (ELISAs)

To optimise parameters, check board titration was carried out in conditions of indirect enzyme immunoassay. The immunoreagents' ratios (antiserum dilutions and concentrations of all synthesised solid-phase antigens) that provided optical density of reaction ranged between 0.8 and 1.2 were determined and then used in competitive indirect ELISA.

The ratio possessing more sensitive TC determination was chosen for the following examinations of assay specificity.

For this purpose, 96-well plates (Costar) were filled with 0.2 ml of conjugate solutions in optimal concentration within 0.05–0.5 μ g/ml in CBB and incubated at 4°C for 16 h. The wells were washed 3–5 times with PBS containing 0.05% Tween 20 (PBS-t). Then 0.1 ml of antiserum in certain dilution in PBS-t with 1% of Gel and standard solutions of antibiotics (10,000–0.1 ng/ml) (B) and (0 ng/ml) (B_0) were added to each well. The plates were left in a humid chamber at room temperature for 1 h and then washed again; the wells were filled with 0.2 ml solution of anti-rabbit IgG antibodies conjugated with horseradish peroxidase. After 1 h incubation and washing, the wells were filled with 0.2 ml of the substrate solution containing 0.4 mg/ml *o*-phenylenediamine and 0.005% hydrogen peroxide in 0.15M

citrate-phosphate buffer, pH 5.0. The enzymatic reaction was stopped after 45 min with 50 μ l of 4M sulphuric acid with 0.1M Na₂SO₃ per well. The optical density was measured at 492 nm using a Dynatech MR 5000 reader (Germany).

The mean optical density in wells ($n=3$) with null TC concentration (B_0) was taken as 100% antibody binding level. The extent of antibody binding for each concentration of TCs ($n=3$) was normalised to 100% control, and antibody binding rate was calculated as:

$$\% \text{ antibody binding} = B/B_0 \times 100$$

The sensitivity of assay was evaluated as 20% inhibition of antibody binding. The cross-reacting of antibodies was assessed as the ratio of TC concentration that induced 50% inhibition of antibody binding to the solid-phase antigen (IC_{50}) to the corresponding LC, CTC, OTC, MeC, MiC and DC concentrations expressed in percentages. The effect of absorbed conjugates on sensitivity and specificity of the test was judged by results of comparison IC_{50} indexes.

Results and discussion

The influence of specially selected solid-phase antigens on cross-reactions between polyclonal antibodies and closely related low-molecular weight substances successfully demonstrated in our previous work (Burkin, 2008) was studied in competitive ELISA using anti-BSA-TC(f) serum and a series of immobilised conjugates. All the TC antibiotics utilised in present work served as haptens when these antigens synthesised.

Among the methods of TC covalent binding with protein molecules formaldehyde condensation tested by several researchers was applied first of all. This reaction formed bond directly with phenyl fragment of TC.

A group of conjugates prepared as a result of interaction between amide group and periodate-oxidised glycoprotein TF that provided alternative steric orientation of the hapten molecule on the carrier appeared to be immunochemically active.

Further structural variety of solid-phase antigens was presented by conjugates on the base of LC. Its methyllysine group in side chain of TC at C₂ made possible to synthesise Gel-LC(ga) and TF-LC(pi) with involving lysine ϵ -NH₂-group and to link LC to protein through carboxyl by activated esters method (Figure 1). Thus, LC-conjugates excepting Gel-LC(f) differed in linking length between carrier and TC nucleus: C9 was for TF-LC(pi), C9+C5 was for Gel-LC(ga) and C5 was for Gel-LC(ae).

The data of interaction between anti-BSA-TC(f) antibodies and all the solid-phase TCs conjugates in the presence of each drug are shown in Table 1(a-c) as half-inhibited binding concentrations (IC_{50}). The degree of cross-reactions determined by ratio between IC_{50} indexes for TC and every analogue was rather high (more than 7%) independently on type of immobilised antigen. This firstly indicates that spatial hapten position on immunogen was such that capable to induce antibody mainly against group epitopes, common region of molecule.

As in previous work (Burkin, 2008) to estimate group specificity of assay, we used coefficient IC_{50MAX}/IC_{50MIN} that means the ratio on concentrations of 50% binding level for the most active and the poorest competitor. It must be noted that maximum coefficient was registered in variant using Gel-TC(f) conjugate which is homologous

Table 1. Cross-reactivity values for tetracyclines and coefficients of group recognition for ELISAs developed on the base of different immobilized antigens.

Immobilised antigens	TC		CTC		OTC		MeC		MiC		DC		LC		GRC ^b
	IC ₅₀ ^a ng/ml	%	IC ₅₀ ng/ml	%	IC ₅₀ ng/ml	%	IC ₅₀ ng/ml	%	IC ₅₀ ng/ml	%	IC ₅₀ ng/ml	%	IC ₅₀ ng/ml	%	
a															
Gel-TC(f)	2.8	100	7.7	36.4	15.3	18.3	30.8	9.1	1.9	147	17.1	16.4	4.5	62.2	16.2
Gel-CTC(f)	2.9	100	3.4	85.3	16.2	17.9	31.6	9.2	3.6	80.6	23.6	12.3	4	72.5	10.9
Gel-OTC(f)	3.2	100	2.6	123	18	17.8	27.7	11.6	2.9	110	17.1	18.7	3.8	84.2	10.7
Gel-MeC(f)	20	100	4.7	426	29.2	68.5	5.5	364	2.9	68.5	15.3	131	14.5	138	10.1
Gel-MiC(f)	18	100	17.1	105	61.8	29.1	52.6	34.2	4.2	429	26.2	68.7	23.6	76.3	14.7
Gel-DC(f)	8.1	100	8.1	100	32.5	24.9	27.7	29.2	5.3	153	26.2	30.9	12.4	65.3	6.1
b															
TF-TC(pi)	6	100	7.1	84.5	23.7	25.3	21.1	28.4	4	150	11.9	50.4	10.6	56.6	5.93
TF-CTC(pi)	2.1	100	2	105	10.6	19.8	8.9	23.6	3	70	8.4	25	4.2	50	5.3
TF-OTC(pi)	22.4	100	16.8	133	66.8	33.5	56.2	39.9	8.9	252	22.4	100	39.8	56.3	7.5
TF-MeC(pi)	4.5	100	3.2	141	32.5	13.8	11.7	38.5	2.6	173	6.9	65.2	7.7	58.4	12.5
TF-MiC(pi)	12.4	100	24.9	49.8	61.8	20.1	153	8.1	22.3	55.6	106	11.7	16.2	76.5	12.3
TF-DC(pi)	11.1	100	6.2	179	34.3	32.4	16.2	68.5	3.6	308	5.9	188	15.3	72.5	9.53
c															
TF-LC(pi)	5.3	100	8.5	62.3	36.2	14.6	32.5	16.3	6.5	81.5	20	26.5	10	53	6.8
Gel-LC(ga)	3.4	100	7.3	46.6	29.2	11.6	42.5	8	11.1	30.6	32.5	10.5	7.3	46.6	12.5
Gel-LC(ae)	12.4	100	9	138	40.2	30.8	24.9	49.8	3.6	344	22.3	55.6	15.3	81	11.2
Gel-LC(f)	3.1	100	8.1	32.3	23.6	13.1	42.5	7.3	5.9	52.5	31.6	9.8	5	62	13.7

^aMean value (*n* = 3). ^bGRC – coefficient of group recognition IC₅₀ MAX/IC₅₀ MIN.

to the immunogen on the type of hapten and conjugating procedure (Table 1a). It is evident that the affinity of antibody binding with 'TC' determinant was higher than with other substances. This explains the greater 'distance' between the representatives of the group. High competitive ability of MiC in this and another ELISAs is probably caused by dimethylamine group of MiC at C₇ position that imitates ambient protein structure nearby the hapten in immunogen.

Alternative hapten position reached by reacting amide of TCs at C₂ with periodate-oxidised glycoside segment of TF made contrary to our expectations no principal changes on sensitivity and group recognition properties (Table 1b). Nevertheless, TF-CTC(pi) was the best to correspond to group-specific assay. The recognition of seven TCs differed only in 5.3 times (Figure 2). When this antigen absorbed determination of TC, CTC and OTC was the most sensitive before the remaining variants. The near values of group specificity can be reached using immobilised TF-TC(pi) and Gel-DC(f) – 5.9 and 6.1, but these variants were less sensitive in 3–4 times.

The LC-conjugates didn't affect significantly on analytical properties of the test (Table 1c). The similar specificity profile and close IC₅₀ values let us to confirm that neither spacer length between hapten and protein nor N-methyllysyl substituent of LC in TF-LC(pi) as opposed to TF-TC(pi) have an influence on antibody binding nature. Immobilised Gel-LC(f) and LC as competitor showed its unexpected worth – side chain of molecule formed by N-methyllysyl residue didn't block antibody binding. The analogous phenomenon was noted by other authors (Pastor-Navarro et al., 2007; R-Biofarm AG). High competitiveness of rolitetracycline (pyrrolidinylmethyl TC) that like LC has N-substituent in amide at C₂ was complained with spontaneous hydrolysis with producing TC.

Thus, estimating the influence of hapten type, its spatial position on the carrier, conjugation method on such test characteristics as sensitivity and specificity (group or selective), it was indicated only combination of these factors did affect on assay parameters determined by anti-BSA-TC(f) serum.

The efficacy of such approach appeared to be moderate, depended probably on quality of antibodies. Nevertheless using of TF-CTC(pi) make group recognition of seven TCs three-fold better (5.3 vs 16.2) in comparison with Gel-TC(f). The

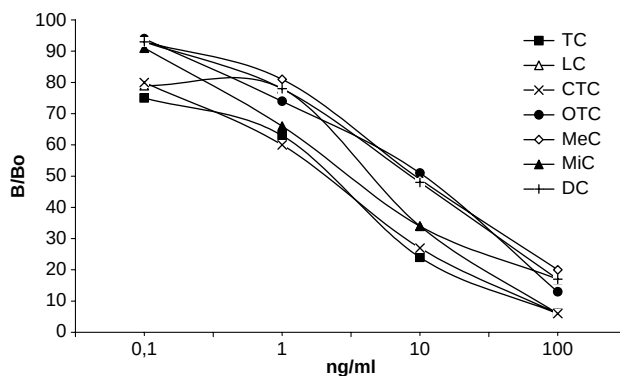


Figure 2. Group-specific assay of tetracyclines based on immobilised TF-CTC(pi) and anti-BSA-TC(f) sera.

sensitivity level reached 0.1 ng/ml level. The structure of selected antigen modified three-dimensional binding with antibodies such way that conditions for better DC and MeC recognition in contrast to described similar tests were set up (Pastor-Navarro et al., 2007; R-Biofarm AG). Thus, such selection procedure may be useful for improvement group specificity properties of polyclonal antibodies-based ELISAs as well as for decreasing cross-reactions.

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