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Analytical Methods

Development of an enzyme-linked immunoassay for the detection of gentamicin in swine tissues

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Abstract

We developed an enzyme-linked immunoassay that provides rapid and sensitive detection of gentamicin in swine tissues. Rabbit was immunized with gentamicin-BSA conjugate and antiserum was collected after the fifth immunization. After optimizing the concentration of immunoreagents, competitive indirect ELISA (ciELISA) gave an IC_{50} value of 0.98 ng/ml, while competitive direct ELISA (cdELISA) exhibited lower IC_{50} value of 0.92 ng/ml, thus cdELISA was further optimized under various pH values and ionic strengths of assay buffer, different coating methods and incubation time. The optimized ELISA can be completed within 45 min and it showed negligible cross-reactivity with other aminoglycosides. The recoveries of gentamicin from spiked swine tissues at levels of 25–200 μg/kg ranged from 64.7% to 101.2% with CVs of 4.5–12.1%, and the detection limits were 6.2 μg/kg in muscle, 3.6 μg/kg in liver and 2.7 μg/kg in kidney, respectively.

Keywords: Gentamicin; Polyclonal antibody; ELISA; Swine tissues

1. Introduction

Gentamicin is a widely used broad spectrum aminoglycoside antibiotic that inhibits the growth of both gram-positive and gram-negative bacteria. It may cause ototoxicity and nephrotoxicity in case of overdosing (Clark, 1977), thus the presence of gentamicin in animal-origin food is potentially hazardous to human health. For consumer protection, the European Agency for the Evaluation of Medical Products (EMEA/MRL/803/01-FINAL, 2001) and the Ministry of Agriculture of China (Official Journal, 2002) have established maximum residue limits (MRLs) for edible tissues and milk (Table 1).

In order to monitor residual gentamicin levels in biological matrixes, a number of analytical methods, mainly microbiological assay (Lantz, Lawrie, Witebsky, & Maclowry, 1980; Ronsner & Aviv, 1980), high-performance liquid chromatography (HPLC) (Al-Amound, Clark, & Chrystyn, 2002; Kaufmamn & Maden, 2005; Posyniak, Zmudzki, & Niedzielska, 2001) and ELISA (Haasnoot et al., 1999; Jin, Jang, Han, & Lee, 2005; Loomans, Wiltenburg, Koets, & Amerongen, 2003), have been developed. Among these methods, the microbiological assay is relatively insensitive and time-consuming. The HPLC assay has high sensitivity but requires extensive cleanup and is therefore not suitable for routine analysis of a large number of samples. In contrast, ELISA is highly sensitive with high throughput and rapid turnaround time, and is therefore advantageous to other methods. While using ELISA for the detection of gentamicin in various biological matrixes has been reported by several groups, most of them are determining gentamicin residue level in milk and serum, in which matrixes interference is relatively low. Haasnoot et al. (1999) developed an effective ELISA for the detection of gentamicin in kidney, however, that assay had the pitfall of having high background readout during detection. This led us to the current study aiming to develop an ELISA that provides rapid detection of gentamicin in swine tissues

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Table 1
Maximum residue limit (μg/kg) of gentamicin in edible tissues and milk

Animal species	Target tissues	EU	China
Pig/cattle	Muscle	50	100
	Fat	50	100
	Liver	200	2000
	Kidney	750	5000
Cattle	Milk	100	200
Chicken	Edible tissues	NE[a]	100

[a] Not established.

including muscle, liver and kidney with high sensitivity and minimized matrix interference.

2. Materials and methods

2.1. Reagents and instruments

Gentamicin sulfate, neomycin sulfate, streptomycin sulfate, dihydrostreptomycin sulfate, kanamycin sulfate and tobramycin sulfate were obtained from National Institute for the Control of Pharmaceutical and Biological products (Beijing, China). Bovine serum albumin (BSA), ovalbumin (OVA), complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium borohydride and HRP were obtained from Beijing Xinjingke Bio. Tec. Co. (Beijing, China). Other reagents were supplied by Beijing Regent Corporation (Beijing, China).

Polystyrene microtiter plates were obtained from Beijing Wanger Bio. Tec. Co. (Beijing, China). ELISA plate reader was purchased from TECAN Inc. (Durham, NC, USA).

2.2. Preparation of immunogen and coating antigens

2.2.1. Carbodiimide (EDC) coupling method

Gentamicin-BSA and Gentamicin-OVA conjugates were synthesized according to the procedure described by Wantanabe, Satake, Kido, and Tsuji (1999).

2.2.2. Glutaraldehyde (GDA) coupling method

Gentamicin sulfate (20 mg) and OVA (20 mg) were dissolved in 10 ml of 0.01 M PBS, the pH value of which was adjusted to 6.5. Then 1.5 ml of freshly prepared 1% glutaraldehyde solution was added drop-wise. After the reaction mixture was gently stirred for 15 min, sodium borohydride was then added to a final concentration of 10 mg/mL, and the solution was incubated for 1 h at 4 °C. Finally, the reaction product was dialysed (over 3 days at 4 °C) against PBS. The immunogen produced by this method was designated as gentamicn-GDA-OVA.

The concentrations of the immunogen and coating antigens were determined by Ultraviolet–visible (UV–vis) spectra photometer (Zhou, Tang, Zhou, & Zhao, 2001).

2.3. Preparation of enzyme tracer

Gentamicin was conjugated with HRP according to the procedure described by Haasnoot et al. (1999) with small modification. Briefly, HRP (5 mg) and gentamicin sulfate (3 mg) were dissolved in 1 ml of PBS (0.01 M, pH 7.4) 1 ml of EDC solution (100 mg/ml) was added drop-wise. After an incubation of 6 h at room temperature, the solution was dialysed against PBS.

2.4. Production of antiserum

Two mg gentamicin-BSA was dissolved in 1 ml of 0.01 M PBS (pH 7.4) and emulsified with 1 ml of Freund's complete adjuvant. The emulsion was injected intracutaneously in the primary immunization. For booster immunizations, 1 mg immunogen was dissolved in 0.5 ml of the above buffer and emulsified with 0.5 ml of Freund's incomplete adjuvant. The emulsion was then injected subcutaneously. The booster immunizations were repeated every three weeks. The rabbit was bled through ear vein one week after each booster injection (since the third immunization). To obtain antiserum, blood samples were left to coagulate for 1 h at room temperature and overnight at 4 °C, followed by centrifugation at 3000*g* for 10 min. The supernatants were carefully collected, divided into aliquots and stored at −20 °C until use.

2.5. ELISA procedure

Two assay formats, indirect ELISA format and direct ELISA format, were used depending on the components (coating antigen or antibody) coated onto the plates. The procedure of indirect ELISA was the same as described by Wang, Zhang, and Shen (2006), and the direct ELISA protocol was similar to that demonstrated by Fitzpatrick, Manning, and O'Kennedy (2004) except TMB instead of OPD was used as substrate. For competitive assays, the concentrations of antibodies and coating antigens or enzyme tracer were optimized by checkerboard titration. Competition curves were obtained by plotting absorbance against the logarithm of analyte concentrations, which were fitted to a four-parameter logistic equation: $y=\{(A-D)/[1+(x/C)^B]\}+D$, where A is the maximum absorbance at no analyte, B is the curve slope at the inflection point, C is the concentration of analyte giving 50% inhibition (IC_{50}), and D is the minimum absorbance at infinite concentration (Li, Qiu, & Wang, 2002). The IC_{50} value was expressed as the sensitivity of ELISA.

2.6. Optimization of ELISA conditions

To develop a rapid and sensitive ELISA, the effect of pH values and ionic strengths of assay buffer, the coating method and incubation time (antigen/antibody reaction) were evaluated. The optimum conditions were determined by B_0 and IC_{50} value.

2.7. Determination of cross-reactivities

Several aminoglycosides were tested for cross-reactivity using the cdELISA procedure described above. The cross-reactivity values were calculated as follows: % cross-reactivity = (IC_{50} of gentamicin/IC_{50} of analytes × 100%) (Li et al., 2002).

2.8. Extraction of samples

Muscle, liver and kidney samples were obtained from pigs that had not been exposed to gentamicin within the previous 6 months. Tissue samples were stored at −20 until analysis. A sample of muscle, liver and kidney (2 g wet mass) was accurately weighed. For recovery study, gentamicin sulfate (1000 ng/ml, prepared in water) was added into tissue samples to produce spiked concentration of 25, 50, 100, and 200 μg/kg.

An aliquot of tissue sample was transferred to a 50 ml polypropylene centrifuge tube. Ten milliliter of 0.2 M PB was mixed with tissue sample. The mixture was then incubated at 60 °C for 30 min. Subsequently, the suspension was centrifuged at 3500*g* for 10 min at room temperature. The supernatant was separated and 100 μl of which was diluted with 900 μl water. Fifty microliter aliquots of the dilution were then pipetted into the microtiter plate for analysis. The recoveries were calculated on the basis of the standard curve constructed by cdELISA.

3. Results and discussion

3.1. Antibody production and characterization

Gentamicin-BSA was used as immunogen for the immunization of rabbit. Gentamicn-OVA and gentamicin-GDA-OVA were used as homologous and heterologous coating antigens respectively to monitor the change of the titer and IC_{50} values of antisera from different bleeds. Despite homologous and heterologous formats, the titers and IC_{50} values remained slightly change since the fourth immunization, therefore, final bleeding was carried out ten days after the fifth immunization. Both higher titers and higher IC_{50} values were obtained using homologous coating antigen than that using heterologous coating antigen. This was probably caused by the recognition of antibodies to cross-linking sites between gentamicin and carrier protein, which resulted in that the displacement of antibodies binding to homologous coating antigens could not be accomplished completely by gentamicin.

3.2. Assay optimization

To evaluate assay formats on ELISA sensitivity, cdELISA and ciELISA were both carried out. After optimizing the concentration of immunoreagents, the cdELISA gave an IC_{50} value of 0.92 ng/ml, while the ciELISA exhibited an IC_{50} value of 0.98 ng/ml using gentamicin-GDA-OVA as coating antigen. The IC_{50} values of the two assay formats were comparable, however, the cdELISA format was more rapid than the ciELISA format, therefore, the cdELISA was further optimized. The results of the effect of various factors on the assay performance of cdELISA are shown in Table 2.

Table 2
Effect of various factors on assay performance of ELISA

Factor	B_0	IC_{50} (ng/ml)
pH		
5.5	1.80	1.04
6.5	2.01	1.04
7.5	2.03	1.13
8.5	2.14	1.15
9.5	1.70	1.26
Ionic strength		
0.025 M PB	1.73	0.93
0.05 M PB	1.40	0.95
0.1 M PB	1.05	1.01
0.2 M PB	0.71	1.14
0.4 M PB	0.45	2.55
Coating method		
4 °C, overnight	1.32	1.08
37 °C, 2 h	1.96	0.96
Coating buffer		
0.01 M PBS, pH 7.4	1.56	0.86
0.05 M CB, pH 9.6	1.84	0.83
Incubation time		
15 min	1.52	0.81
30 min	1.70	0.87
45 min	1.88	0.89
60 min	1.82	0.92

3.2.1. Effect of pH

Though relatively low B_0 value was observed at pH 5.5 and 9.5, variation of assay pH value did not show significant effect on assay performance, which indicated the assay

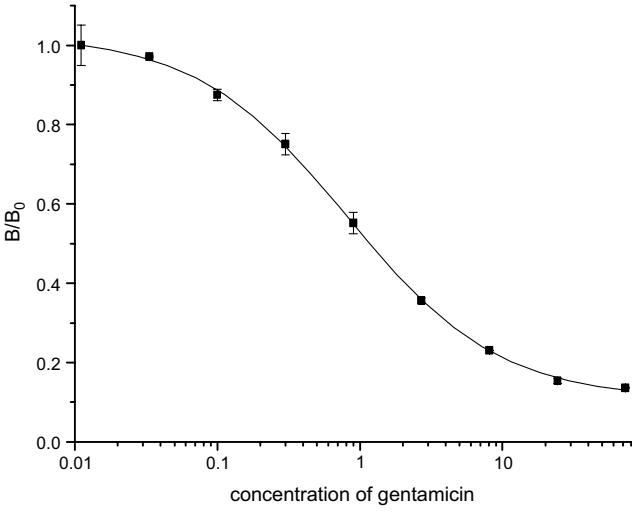


Fig. 1. Standard curve of competitive direct ELISA for gentamicin. Each point presents the average of four well replicates, and error bars represent standard deviations.

Table 3
Cross-reactivities of gentamicin ELISA with different aminoglycosides

Aminoglycosides	Cross-reactivity (%)
Gentamicin	100
Kanamycin	<0.1
Tobramycin	<0.1
Neomycin	<0.1
Streptomycin	<0.1
Dihydrostreptomycin	<0.1

and the antibody was robust to pH change. The lowest IC_{50} value of 1.04 ng/ml was obtained at pH 5.5 and 6.5, and pH 6.5 was selected because of its higher B_0 value.

3.2.2. Effect of ionic strength

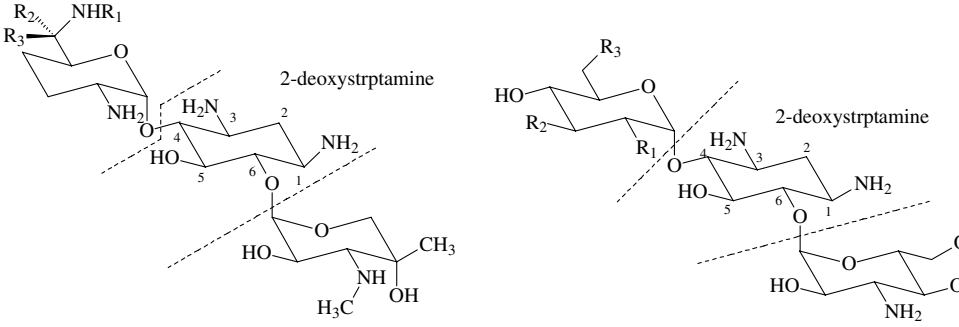
Decreasing ionic strength from 0.4 M to 0.025 M phosphate buffer (PB) resulted in higher B_0 value and lower IC_{50}, indicating that lower ionic strength is more benefit for antigen/antibody reaction, thus low ionic strength rather than high ionic strength was maintained during the assay.

3.2.3. Effect of coating method

Coating antibody at 37 °C for 2 h gave lower IC_{50} value and higher B_0 value than coating at 4 °C overnight, thus coating method of 37 °C for 2 h was selected. Carbonate buffer (CB) is better than phosphate buffer saline (PBS) when coating buffer was selected since the former showed greater color development with slightly higher sensitivity.

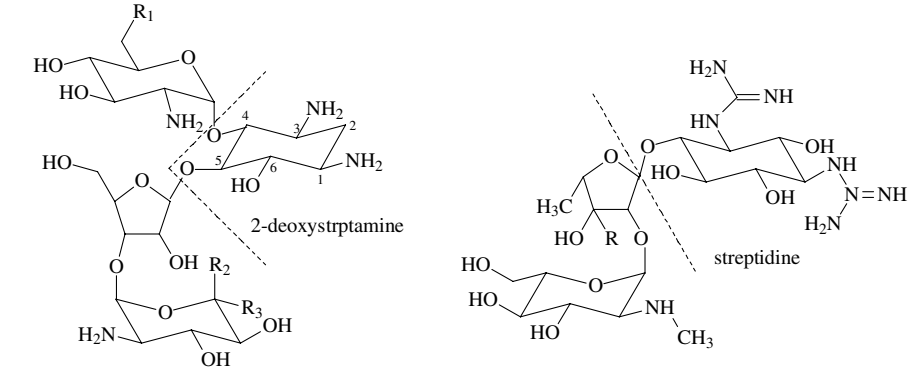
3.2.4. Effect of incubation time

Incubation of antigen and antibody for 15 min showed the lowest IC_{50} value of 0.81 ng/ml, although relatively low B_0 value was observed. Considering the rapidity of manipulation, incubation time was chosen at 15 min. In previous studies, the antigen/antibody incubation time was 1 h, thus the ELISA procedures, excluding time for coating and blocking, required about 90 min for competitive direct ELSIA format (Haasnoot et al., 1999; Jin et al., 2005) and even longer for competitive indirect ELSIA format (Loomans et al., 2003). Compared with these ELISA methods, the analysis time of the present



	R_1	R_2	R_3
gentamicin C_1	CH_3	H	CH_3
gentamicin C_{1a}	H	H	H
gentamicin C_2	H	H	CH_3
gentamicin C_{2a}	H	CH_3	H
gentamicin C_{2b}	CH_3	H	H

	R_1	R_2	R_3
kanamycin A	OH	OH	NH_2
kanamycin B	NH_2	OH	NH_2
kanamycin C	NH_2	OH	OH
tobramycin	NH_2	H	NH_2



	R_1	R_2	R_3
neomycin B	NH_2	CH_2NH_2	H
neomycin C	NH_2	H	CH_2NH_2

	R
streptomycin	CHO
dihydrostreptomycin	CH_2OH

Fig. 2. Chemical structures of aminoglycosides.

ELISA was reduced to 45 min by shortening the antigen/antibody incubation time. With this tremendous saving in time, our rapid ELISA provides higher analysis efficiency for the determination of gentamicin than conventional ELISA.

On the basis of these results, the standard curve for gentamicin ELISA was drawn as seen in Fig. 1.

3.3. Cross-reactivity

The gentamicin ELISA was very specific and had little cross-reactivity with several usual-used aminoglycosides (Table 3). The specificity of this ELISA can be explained by the differences in molecular structure of aminoglycosides (Fig. 2). They all consist of two or more aminosugars linked by glycosidic bonds to a cyclitol component, which is either streptidine (found in streptomycin) or 2-deoxystreptamine (found in neomycin, gentamicin and kanamycin). The kind of 2-deoxystreptamine aminoglycosides can be further divided into three subclasses according to the pattern of substitution on cyclitol. The gentamicin and kanamycin subclasses are 4,6-disubstituted deoxystreptamines, while the neomycin subclasses is 4,5-disubstituted deoxystreptamines. The aminosugars attached to cyclitol also differ in numbers and structure (Stead, 2000). Therefore, the great structure differences result in the high assay specificity of gentamicin ELISA.

3.4. Sample extraction

Haasnoot et al. (1999) used trichloroacetic acid (TCA) to extract gentamicin from kidney, however, significant matrix interference was observed (1999). This phenomenon was also found in our study when using TCA as extraction reagents. The matrix interference lowered the color development and thus caused high background level and unacceptable recovery. In contrast, when 0.2 M phosphate buffer was applied to extract gentamicin from swine tissues at 60 °C for 30 min, low background level and good recoveries was obtained. The recoveries results are shown in Table 4. Recoveries of gentamicin in muscle ranged from 89.8% to 101.2% with CVs of 6.1–10.1%, recoveries of gentamicin in liver ranged from 76.6% to 84.3% with CVs of 4.5–8.3% and recoveries of gentamicin in kidney ranged from 64.7 to 74.7% with CVs of 5.7–12.1%. The detection limits, which was defined as the mean background level plus $3 \times SD$, was 6.2 μg/kg in meat, 3.6 μg/kg in liver and 2.7 μg/kg in kidney respectively ($n = 4$).

Table 4
Recoveries of gentamicin from spiked swine tissues ($n = 4$)

Sample	Spiked level (μg/kg)	Recovery (%)	CV (%)
Muscle	25	101.2	10.1
	50	98.9	9.3
	100	89.8	6.1
	200	94.5	8.7
Liver	25	82.5	7.4
	50	84.3	8.3
	100	80.1	4.5
	200	76.6	7.7
Kidney	25	74.7	10.3
	50	68.9	7.8
	100	71.3	12.1
	200	64.7	5.7

4. Conclusion

In this study, we developed a rapid and sensitive ELISA for gentamicin in swine tissues. The time required for the analysis, except for coating and blocking, was less than 45 min. The detection limits, which ranged from 2.7–6.2 μg/kg in different tissues, were far below current MRLs established by the EU and China. The recoveries and deviations of gentamicin from spiked swine tissues were also within acceptable range. Thus the ELISA developed herein can be used as a convenient tool for the rapid detection of gentamicin in swine tissues.

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