JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

# Rapid Enzyme-Linked Immunosorbent Assay and Colloidal Gold Immunoassay for Kanamycin and Tobramycin in Swine Tissues

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A monoclonal antibody (Mab) was produced by using the kanamycin−glutaraldehyde−bovine serum albumin (Kan−GDA−BSA) conjugate as the immunogen. The anti-Kan Mab exhibited high cross-reactivity with tobramycin (Tob) and slight or negligible cross-reactivity with other aminoglycosides. The specificity and cross-reactivity of this Mab are discussed regarding the three-dimensional, computer-generated molecular models of the aminoglycosides. Using this Mab, a rapid enzyme-linked immunosorbent assay (ELISA) and a colloidal gold-based strip test for Kan and Tob were developed. The rapid ELISA showed a 50% inhibition value ($IC_{50}$) of 0.83 ng/mL for Kan and 0.89 ng/mL for Tob with the analysis time less than 40 min, and the recoveries from spiked swine tissues at levels of 25–200 $\mu$g/kg ranged from 52% to 96% for Kan and 61% to 86% for Tob. In contrast, the strip test for Kan or Tob had a visual detection limit of 5 ng/mL in PBS, 50 $\mu$g/kg in meat or liver, and 100 $\mu$g/kg in kidney, and the results can be judged within 5–10 min. Observed positive samples judged by the strip test can be further quantitated by ELISA, hence the two assays can complement each other for rapid detection of residual Kan and Tob in swine tissues. Moreover, physical-chemical factors that affected the ELISA and strip test performance were also investigated. The effect of pH and antibody amount for gold−antibody conjugation on the strip test sensitivity was determined followed by a theoretical explanation of the effects.

**KEYWORDS: Kanamycin; tobramycin; monoclonal antibody; ELISA; strip test; molecular modeling**

## INTRODUCTION

Kanamycin (Kan) and tobramycin (Tob) (**Figure 1**) are both aminoglycoside antibiotics that inhibit the growth of both Gram-positive and Gram-negative bacteria (*1*). Overdosing of kanamycin and tobramycin can cause ototoxicity and nephrotoxicity (*2, 3*), thus the presence of Kan and Tob in food of animal origin is potentially hazardous to human health. For consumer protection, the European Union (EU) has established maximum residue limits (MRLs) for Kan in edible tissues and milk: 100 $\mu$g/kg for meat, 600 $\mu$g/kg for liver, 2500 $\mu$g/kg for kidney, and 150 $\mu$g/kg for milk (*4*). Although Tob has significant toxicity effects, the MRLs for it currently have not been established.

To monitor Kan and/or Tob level in biological matrixes, a number of analytical methods, such as high-performance liquid chromatography (HPLC) (*5–7*), capillary electrophoresis (CE) (*8*), and immunoassays (*9–16*), have been developed. As HPLC and CE methods require expensive instrumentation, highly skilled personnel, and extensive sample cleanup, they are not suitable for routine analysis of a large number of samples. For screening purposes, immunoassay is advantageous to instrument method because of its high sensitivity and specificity, high throughput, and rapid turnaround time. In previous studies, Kitagawa et al. (*10*), Watanabe et al. (*12*), and Jin et al. (*15*) reported development of immunoassays for determining kanamycin in serum or milk. However, the polyclonal or monoclonal antibodies they obtained were specific to kanamycin and had only slight or negligible cross-reactivity with other aminoglycosides. Besides, the detection of Kan or Tob in a more complex matrix such as animal tissues has not been reported. Here we prepare an anti-Kan monoclonal antibody with high cross-reactivity to Tob and develop an enzyme-linked immunosorbent assay (ELISA) that provides simultaneous quantitative detection of Kan and Tob in swine tissues.

However, for a more simple and rapid qualitative detection, a one-step strip test would be a better choice. Compared with the ELISA, it has several advantages: sample pretreatment can be further simplified; results can be obtained within 5–10 min; and all of the reagents are included in the strip without the need for any expensive equipment. Since Verheijen et al. introduced this technique to detect sulfadimidine (*17*) and

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10.1021/jf703602b CCC: $40.75 
Published on Web 04/05/2008

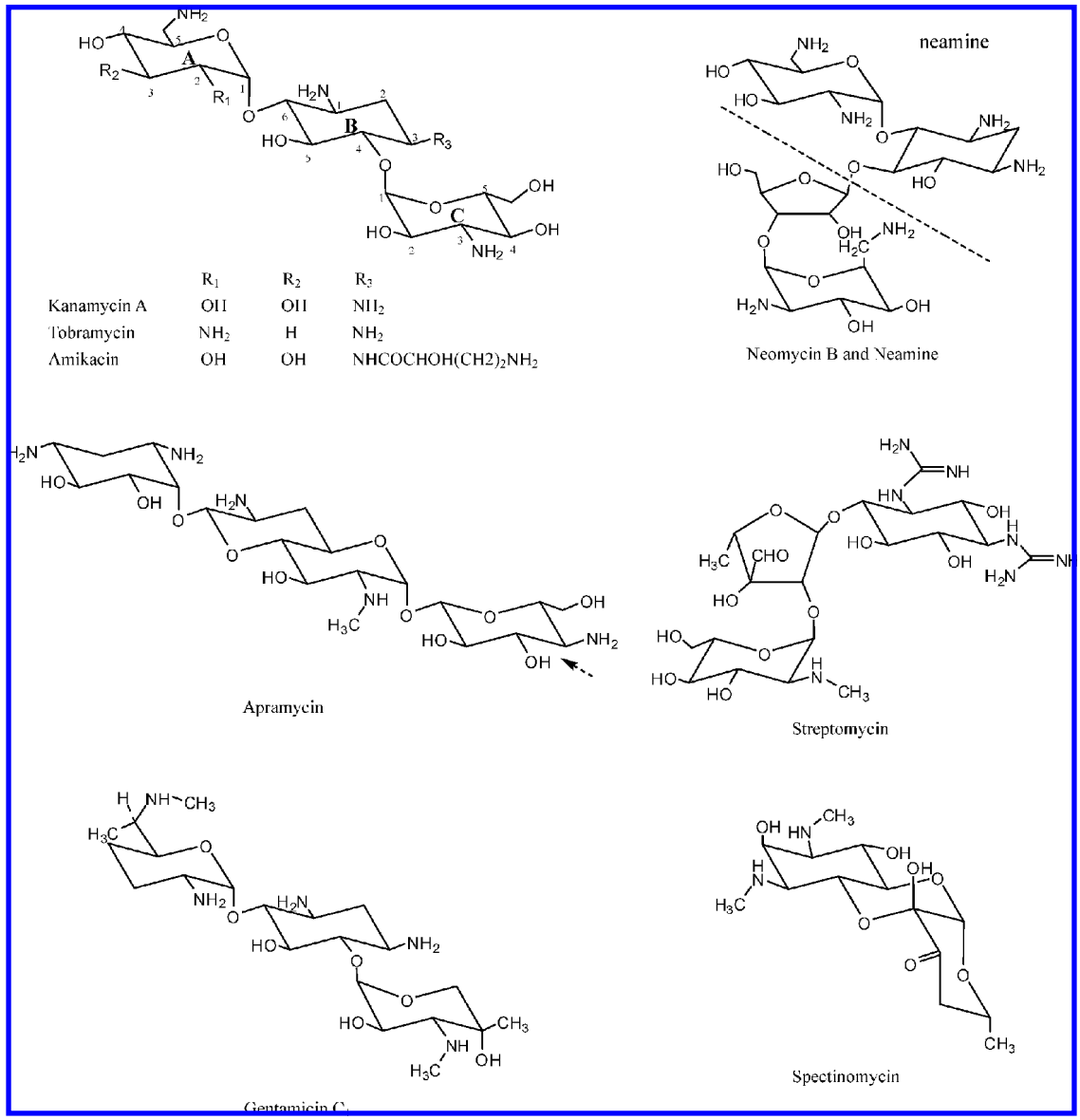


**Figure 1.** Chemical structures of aminoglycosides.

streptomycin residues (*18*) in urine and milk samples, many researchers have adopted this method to detect various small mass substances, e.g., mycotoxins (*19–21*), pesticides (*22–24*), and veterinary drugs (*15, 25–29*). However, among these studies, little attention has been paid to the optimization of gold−antibody conjugation conditions (pH value and antibody amount). In this study, we investigate the effect of these conditions on the sensitivity of a strip test. Moreover, we also make a presumption on this effect.

Molecular modeling can provide useful information in relation to the physicochemical properties of chemicals and can assist in designing haptens (*30, 31*) or explaining the cross-reactivity of an antibody (*32–35*). A previous study conducted in our laboratory has employed molecular modeling to examine the relationship between chemical structures of fluoroquinolones and their antibody affinity (*36*). In this study, the aminoglycoside analogues are modeled using the semiempirical quantum method AM1 and Hartree–Fock method in an effort to explain the observed cross-reactivity.

## MATERIALS AND METHODS

**Chemicals and Materials.** Kanamycin (Kanamycin A > 95%), tobramycin, amikacin, gentamicin, neomycin, streptomycin, and neamine were obtained from the National Institute for the Control of Pharmaceutical and Biological products (Beijing, P.R.C.). Spectinomycin and apramycin were purchased from the China Institute of Veterinary Drug Control (Beijing, P.R.C.). Bovine serum albumin (BSA), ovalbumin (OVA), complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), goat antimouse IgG, goat antimouse IgG−horseradish peroxidase (HRP) conjugate, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), glutaraldehyde (GDA), and chlorauric acid (tetrachloroauric acid trihydrate, $HAuCl_4 \cdot 3H_2O$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium borohydride, HRP, and polyethylene glycol 1500 (PEG 1500) were obtained from Beijing Xinjingke Bio. Tec. Co. (Beijing, P.R.C.). RPMI 1640 medium, fetal bovine serum (FBS), hypoxanthine−aminopterin−thymidine (HAT) medium, and hypoxanthine−thymidine (HT) medium were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Purified water was obtained using a Milli-Q water purification system (Millipore, Bedford, MA). Other reagents were purchased from Beijing Regent Corporation (Beijing, P.R.C.). A Hi-Trap protein G column was obtained from Amersham Pharmacia Biotech, Inc. (Uppsala, Sweden). A mouse monoclonal antibody isotyping kit was purchased from Pierce Biotechnology, Inc. (Rockford, Il, USA). Nitrocellulose membrane (AE99, AE100, FF85, and Prima85), glass fiber membrane (Glass 33), and absorbance pad (CF4) were supplied by Whatman International, Ltd. (Middlesex, U.K.). Microtiter plates, microculture plates, and cell culture bottles were obtained from Costar Group, Inc. (Bethesda, MD, USA).

**Apparatus.** The ELISA plate reader was from TECAN U.S, Inc. (Durham, NC, USA). The ZX1000 Dispensing Platform and CM4000 Guillotine Cutting Module used to prepare test strips were purchased from BioDot Inc. (Irvine, CA, USA).

**Preparation of Immunogens and Coating Antigens.** *Carbodiimide (EDC) Coupling Method.* Kan−BSA and Kan−OVA conjugates were synthesized according to the procedure described by Wantanabe et al. (*12*).

*Glutaraldehyde (GDA) Coupling Method.* The Kan−carrier protein conjugate was synthesized as follows: Kanamycin sulfate (20 mg) and BSA or OVA (20 mg) were dissolved in 10 mL of 0.01 M phosphate buffer saline (PBS), the pH value of which was adjusted to 6.5. Then 1.5 mL of freshly prepared 1% glutaraldehyde solution was added dropwise. After the reaction mixture was gently stirred for 15 min, sodium borohydride was 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 (0.01 M, pH 7.4). The conjugates produced by this method were designated as Kan−GDA−BSA and Kan−GDA−OVA, respectively.

*Preparation of Enzyme Tracer.* The Kan−HRP conjugate was synthesized according to the procedure demonstrated by Haasnoot et al. (*37*).

**Production of Mab Against Kan.** *Immunization of Mice.* Twenty BALB/c female mice (8 weeks old) were immunized with Kan−BSA or Kan−GDA-BSA conjugates. The first dose consisted of 100 $\mu$g of immunogen for injection subcutaneously as an emulsion of PBS and Freund's complete adjuvant. Two subsequent injections were given at two-week intervals with the same dosage of immunogen emulsified in Freund's incomplete adjuvant. Antisera were collected one week after the third immunization and were screened for anti-Kan activity with the ELISA described below. The mouse showing the highest anti-Kan activity received a fourth injection intraperitoneally (i.p.). Four days later, the spleen of the injected mouse was removed for hybridoma production.

*Hybridoma Production, Selection, and Cloning.* Cell fusion procedures were carried out according to the procedure described by Köhler and Milstein (*38*) with some modifications. Briefly, mouse spleen lymphocytes were fused with myeloma cells at a 5:1 ratio using PEG 1500 as the fusing agent. The fused cells were suspended in HAT-RPMI 1640 medium (supplemented with 20% fetal calf serum) and then distributed to five 96-well microculture plates, which were previously incubated with a feeder layer of peritomeal macrophages. Eleven days after the fusion, cell-free culture supernatants were determined for the presence of anti-Kan antibody using a combination of noncompetitive and competitive indirect ELISA (ciELISA). Well cultures resulting in high OD values and showing significant Kan recognition activity were selected for cloning by limiting dilution using HT-RPMI 1640 medium (supplemented with 20% fetal calf serum). Stable antibody producing clones were expanded in RPMI 1640 medium (supplemented with 20% fetal calf serum) and cryopreserved in liquid nitrogen.

*Production, Purification and Characterization of Monoclonal Antibody.* A mature female BALB/c mouse was injected (i.p.) with 0.5 mL of paraffin 7 days before receiving an i.p. injection of the hybridoma cells ($1 \times 10^7$ cells) suspended in RPMI 1640 medium. Ascites fluid was collected 10 days after the injection and then stored at –20 °C until use. Purification of Mab was achieved by saturated ammonium sulfate precipitation followed by affinity chromatography on a protein G column (*39*). The class and subclass of the isotypes of the purified antibody were determined by using a mouse monoclonal antibody isotyping kit.

**Cross-Reactivity and Molecular Modeling.** Several aminoglycosides including amikacin, apramycin, gentamicin, neamine, neomycin, spectinomycin, streptomycin, and tobramycin were tested for cross-reactivity using the competitive direct ELISA (cdELISA) described below. The cross-reactivity values were calculated as follows

$$\text{percent cross-reactivity} = \frac{IC_{50}(\text{Kan, pmol})}{IC_{50}(\text{analytes, pmol})} \times 100$$

For all the molecules tested, the 2D molecule formulas were drawn in Chemdraw program and the 3D models were constructed in Chem3D program in the Chemoffice Ultra 10 software package (Cambridgesoft Corporation, MA, USA). Calculations of all molecules were performed using the Gaussian 03 interface in Chemoffice Ultra 10. To save computational time, initial geometry optimizations were carried out with the molecular mechanics (MM), using the MM2 force fields. The lowest-energy confirmations of the molecules obtained by the MM2 method were further optimized by semiempirical AM1 method. Their fundamental vibrations were also calculated using the same AM1 method to check if they were true minima. The charge of each molecule was calculated using the Hartree–Fock method with a 6-31G basis set. The Connolly surface of all molecules was calculated in Chem3D, and mapping was colored by electrostatic potential energy.

**ELISA Procedure.** Two assay formats, indirect ELISA and direct ELISA, were carried out depending on the components (coating antigen or antibody) coated onto the plates. The procedure of indirect ELISA was the same as previously used in our laboratory (*40*), and the direct ELISA procedure was performed as follows: polystyrene microtiter plate wells were coated with 100 $\mu$L of anti-Kan monoclonal antibody (1:10000 in 0.05 M carbonate buffer) at 37 °C for 2 h. After coating, plates were blocked for 2 h at 37 °C with 200 $\mu$L per well of blocking solution (0.01 M PBS, containing 0.5% casein). The solution was then discarded, and plates were washed four times with washing solution (0.01 M PBS, containing 0.05% Tween 20). Then, 50 $\mu$L per well of analytes (Kan or Tob) followed by 50 $\mu$L per well of Kan-HRP conjugate (1:6000 in phosphate buffer, PB) were added and incubated for 15 min at 37 °C. After another washing step, the color development was initiated by adding 100 $\mu$L of the substrate/chromogen solution (TMB/$H_2O_2$ in acetate buffer, pH 5.5). The solution was incubated for 15 min at 37 °C before the enzymatic reaction was stopped by adding 2 N $H_2SO_4$ (50 $\mu$L/well). The optical density (OD) of each well was measured at 450 nm by an enzyme immunoassay reader. Competition curves were obtained by plotting absorbance against the logarithm of analyte concentrations. The software package OriginPro 7.0 (OriginLab Corp., MA. USA) was used to calculate the four-parameter sigmoidal curve 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. The $IC_{50}$ value was expressed as sensitivity of ELISA.

**One-Step Strip Preparation and Test Procedure.** *Preparation of Colloidal Gold.* Colloidal gold was prepared as described by Zhou et al. (*22*). Briefly, 100 mL of 0.01% (m/v) chlorauric acid was heated to boiling, and then 2.0 mL of 1% trisodium citrate was added under constant stirring. After boiling for 15 min with stirring, the solution was left to cool, and deionized water (about 30 mL) was added to the initial volume. Supplemented with 0.05% sodium azide, the obtained colloidal gold suspensions could be stored at 4 °C for several months.

*Preparation of Detection Reagents.* The purified anti-Kan Mab was dialyzed against distilled water for 24 h at 4 °C. Before conjugation with the colloidal gold, the optimal pH value and antibody concentration were determined to obtain the best sensitivity by checkboard titration. With gentle stirring, 10 mL of colloidal gold solution was adjusted to pH 7.0 with 0.1 M $K_2CO_3$ or 0.1 M HCl, and then 50 $\mu$g of purified anti-Kan Mab was added dropwise. After incubation at room temperature for 15 min, 3 mL of 5% BSA solution was added, and stirring was continued for another 15 min. The mixture was centrifuged at 12000 rpm for 15 min, and the precipitate of the gold-labeled antibodies was resuspended in 5 mL of dilution buffer (0.01 M PBS, containing 1% sucrose and 0.5% Triton-100, pH 7.2) and stored at 4 °C. Gold-labeled anti-Kan Mab (detection reagent) was dispensed onto a conjugate pad (0.5 $\mu$L per $mm^2$; glass fiber membrane, Glass 33, Whatman) and then dried for 1 h at 37 °C.

*Immobilization of Capture Reagents.* The goat antimouse IgG (2 mg/mL) and Kan−GDA−OVA (1 mg/mL) were applied to the nitrocellulose membrane as the control line and test line, respectively. The dispensed volumes were both 0.3 $\mu$L per mm line. After dispensation, the nitrocellulose membrane was dried for 45 min at 37 °C and stored under dry conditions at room temperature until use.

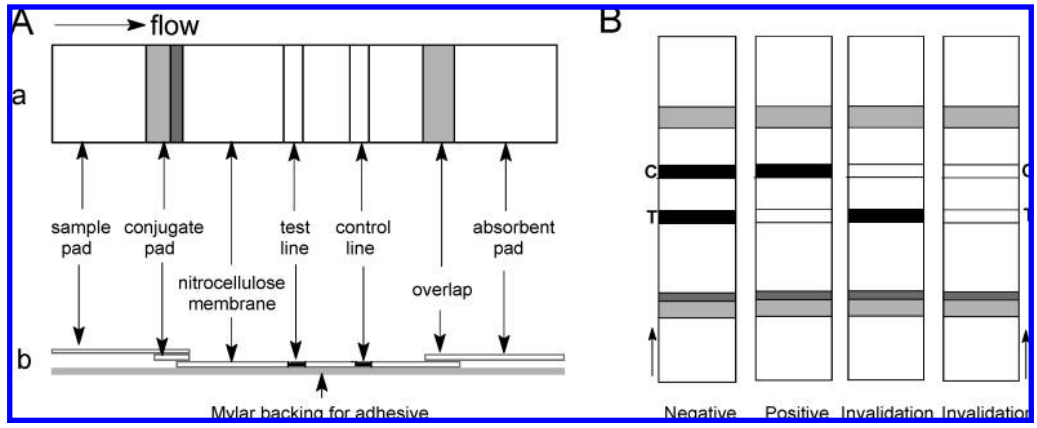


**Figure 2.** (**A**) Schematic description of the one-step strip. a: Top view. b: Cross-section. A complete one-step strip test device consists of a test strip as shown here, packed in a plastic housing. (**B**) Illustration of strip test results. C and T represent control line and test line, respectively.

**Table 1.** Cross-Reactivity of Kan Mab with Different Aminoglycosides

| aminoglycosides | $IC_{50}$ (ng/mL) | $IC_{50}$ (pmol/mL) | cross-reactivity (%) |
|---|---|---|---|
| Kanamycin | 0.83 | 1.72 | 100 |
| Tobramycin | 0.89 | 1.91 | 90 |
| Apramycin | 32 | 54.7 | 3.1 |
| Amikacin | 345 | 672.5 | 0.3 |
| Neamine | >10000 | >10000 | <0.01 |
| Neomycin | >10000 | >10000 | <0.01 |
| Gentamicin | >10000 | >10000 | <0.01 |
| Streptomycin | >10000 | >10000 | <0.01 |
| Spectinomycin | >10000 | >10000 | <0.01 |

*Assembly of the Strip.* The strip assembly procedure was similar to that described by Zhou et al. (*22*) with modifications. Briefly, the nitrocellulose membrane (AE99, AE100, FF85, and Prima85, Whatman)lined with capture reagents was pasted on the center of the plastic backing, and then the conjugate pad dispensed with the detection reagent was attached on it so that it laid over (2 mm) the nitrocellulose membrane. The sample pad (Glass 33, Whatman) was also pasted with one end overlapping the conjugated pad. The absorbent pad (CF4, Whatman) was pasted on the other side of the backing so that it also laid over (2 mm) the nitrocellulose membrane. The assembled plate was cut to the appropriate size (4 mm width) for making the one-step strip. Finally, the strip was mounted in a plastic housing for use. A schematic description of the colloidal gold-based one-step strip is illustrated in **Figure 2**.

*Test Procedure.* Four drops (about 200 $\mu$L) of standard solution or sample extract were added onto the sample pad, and the solution migrated toward the absorbent pad. After 5 min, the result could be read by the naked eyes. When Kan or Tob was absent in the sample, the detection reagent would be trapped by capture reagent to form a visible test line. When Kan or Tob was present in the sample, they would compete with the immobilized capture reagent for the limited amount of detection reagent. If sufficient Kan or Tob molecules existed, they would then hinder the detection reagent from combining with the capture reagent, thus a positive sample gave no visible test line on the nitrocellulose membrane. When the test procedure was properly carried out, the control line was always visible.

**Sample Preparation.** Meat, liver, and kidney samples were obtained from pigs that had not been exposed to Kan or Tob. Tissue samples were stored at –20 °C until analysis. A homogenized sample of meat, liver, or kidney (1 g wet mass) was accurately weighed. For recovery study, Kan or Tob standard solution (1000 ng/mL, prepared in 0.01 M PBS) was added into homogenized tissue samples to produce spiked concentrations of 25, 50, 100, and 200 $\mu$g/kg.

*Sample Pretreatment for ELISA.* An aliquot of tissue sample was transferred to a 50 mL polypropylene centrifuge tube. After 5 mL of 3% trichloroacetic acid solution was added, the sample was mixed briefly and centrifuged at 4000 rpm for 10 min to deproteinate. The supernatant was separated and neutralized with 30% NaOH solution. Then 100 $\mu$L of this solution was diluted with 900 $\mu$L of the optimized assay buffer, of which a 50 $\mu$L aliquot per well was pipetted into the microtiter plate for analysis. The recoveries of Kan and Tob were both calculated on the basis of the Kan standard curve constructed by cdELISA.

*Sample Pretreatment for Strip Test.* An aliquot of tissue sample was transferred to a 50 mL polypropylene centrifuge tube. An aliquot of 10 mL of 0.01 M PBS (pH 7.2) was mixed with the tissue sample, and the mixture was shaken for 2 min. After standing for 5 min, four drops (about 200 $\mu$L) of the upper suspension were pipetted onto the sample pad of the one-step strip for analysis.

## RESULTS AND DISCUSSION

**Hybridoma Production and Antibody Characterization.** Kan−BSA and Kan−GDA−BSA conjugates were used as immunogens for the immunization of mice, while Kan−OVA and Kan−GDA−OVA were coated onto ELISA plates to determine the titer and inhibition level of antisera. Seven of the ten mice immunized with Kan−BSA and six of the ten mice immunized with Kan−GDA−BSA produced antisera with significant anti-Kan activities. Both higher titers and higher $IC_{50}$ values of antisera were obtained in homologous format than that of antisera in heterologous format. This probably resulted from the recognition of antibodies to cross-linking sites between Kan and the carrier protein, which caused that the displacement of antibodies binding to homologous coating antigens could not be accomplished completely by Kan (*40*). The mouse immunized with Kan−GDA−BSA showed high titer (1:10 000) and the lowest $IC_{50}$ values (5.5 ng/mL) in heterologous format, thus it was chosen for subsequent experiments. After cell fusion and cloning, six hybridomas with the ability to excrete anti-Kan antibody were obtained. The most sensitive hybridoma named 5E8-B9 showed the $IC_{50}$ value of 0.75 ng/mL. This means that the sensitivity of Mab increased approximately 7-fold in comparison to that of the antiserum tested above. Compared with the antibodies prepared by other groups (*10, 12–14*), the monoclonal antibody we developed is more sensitive. Using a mouse monoclonal antibody isotyping kit, 5E8-B9 was determined to be a IgG1 isotype with a kappa light chain, and this hybridoma was used for further evaluation of Mab specificity and subsequent immunoassay development.

**Cross-Reactivity and Molecular Modeling Studies.** The results of cross-reactivity demonstrate that this Mab has high cross-reactivity with Tob (90%), slight cross-reactivity with apramycin (3.1%) and amikacin (0.3%), and negligible cross-reactivity with other aminoglycosides (<0.01%) (**Table 1**). As these results were quite different from that reported by Kitagawa et al. (*10*) and Watanabe et al. (*12*), molecular modeling was

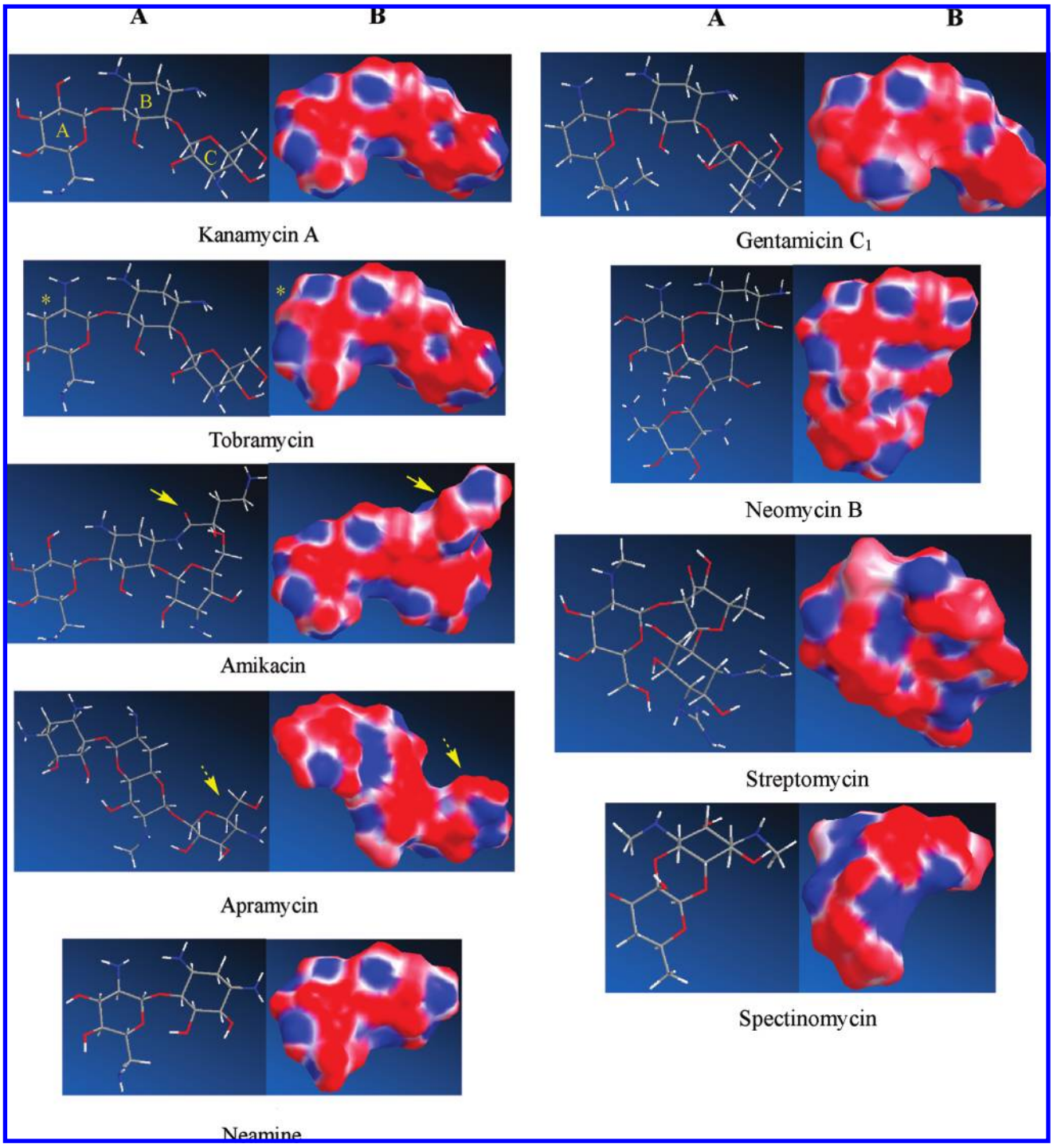


**Figure 3.** (**A**) Stick models of the minimum energy conformations of the aminoglycosides. The elements are represented in the following manner: oxygen, red; nitrogen, blue; hydrogen, white; and carbon, off white. (**B**) Connolly surface mapping colored by electrostatic potential of the aminoglycosides. Red, negative charge; blue, positive charge; and white, neutral charge.

then performed to explain the observed cross-reactivity in a three-dimensional degree.

The minimum energy conformations and Connolly surface mapping colored by electrostatic potential of the aminoglycosides (**Figure 1**) are shown in **Figure 3**. Kan and Tob are almost identical with respect to surface charge and structure of B and C rings. The only structural difference between the Kan and Tob lies in the substitution of the A ring (**Figure 3**, asterisks), where the hydroxyl groups at position 2 and 3 in Kan are replaced by an amino group and a hydrogen in Tob, respectively. In contrast, Kan and amikacin show uniform surface charge and structure on the A and B rings. The substitution of the $NH_2$ group in Kan by the large $NHCOCHOH(CH_2)_2NH_2$ group in amikacin at position 3 on the B ring introduces steric hindrance to the right portion of amikacin (**Figure 3**, plain arrows). Based on these observations, it is assumed that the Kan antibody prepared by Kitagawa et al. may bind primarily to the A ring of Kan, and the hydroxyl groups at position 2 and 3 may play an important role in antibody binding, since this antibody has high cross-reactivity with amikacin (26.7%) and negligible cross-reactivity with Tob (0.0667%). As the Mab against Kan obtained by Watanabe et al. showed slight cross-reactivity with tobramycin (5.3%) and amikacin (0.65%), it appears that the majority of Kan is involved in binding by this Mab, and the low affinity

with both Tob and amikacin suggest that several contact points may contribute to the recognition by this Mab. In contrast, the Mab we obtained has high affinity with Tob and low affinity with amikacin. This suggests that the C ring of Kan may be the main binding portion of this Mab and the introduction of steric hindrance by the $NHCOCHOH(CH_2)_2NH_2$ group disturbs the antibody binding to amikacin.

It is not expected that the affinity of Mab to apramycin is only 38-fold lower than that of Kan because there are many heterologous moieties between the two molecules, especially one more six-membered ring existing in the apramycin molecule. This result may be attributed to the structure and electronic similarity of the right portion of apramycin compared to that of Kan (**Figure 3**, dashed arrows), since that portion of the two molecules differs only in the opposite substitution position of the amino and hydroxyl groups (**Figure 1**, dashed arrow). Minimum energy confirmation and electrostatic potential of neamine have close fits with the A and B rings of Kan; however, it was not bound by this Mab. It further suggests that the C ring of Kan is the main antibody binding portion. According to this result, the Mab we produced would probably recognize the three components of Kan (Kan A, Kan B, and Kan C) very identically since they have the same C ring. Although gentamicin shows a high degree of structural similarity with Kan, the electrostatic potentials are remarkably distinct due to the different substitutions on the rings of gentamicin and Kan, thus gentamicin cannot be recognized by Kan Mab. Similarly, it is not surprising that no cross-reactivity with neomycin, streptomycin, and spectinomycin was observed since the volumes, shapes, and also the electrostatic potentials of these compounds are dramatically different from that of Kan.

**ELISA Optimization.** To evaluate the effect of assay formats on ELISA sensitivity, cdELISA and ciELISA were both carried out. After optimizing the concentration of immunoreagents by checkerboard titration, the cdELISA for Kan gave an $IC_{50}$ value of 0.86 ng/mL, while the ciELISA exhibited an $IC_{50}$ value of 0.73 ng/mL using Kan−GDA−OVA as the 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, it was further optimized.

The effect of pH and ionic strength of assay buffer on cdELISA performance was investigated. Neutral pH value and lower ionic strength were found to be more beneficial to antigen/antibody reaction with a higher $B_0$ value ($B_0$ is the absorbance of the sample without Kan) and lower $IC_{50}$ (**Table 2**), hence pH 7.0 and low ionic strength (0.025 M phosphate buffer, PB) were maintained during the assay.

Coating the antibody at 37 °C for 2 h gave a higher $B_0$ value than coating at 4 °C overnight, while $IC_{50}$ values were similar (**Table 2**). Thus, the coating method of 37 °C for 2 h was selected. PBS (pH 7.4) is better than carbonate buffer (CB, pH 9.6) when coating buffer was selected since the former showed greater color development with slightly higher sensitivity (**Table 2**).

Compared to reaction for 30, 45, or 60 min, incubation of antigen and antibody for 15 min showed no significant change in $B_0$ value and $IC_{50}$ value (**Table 2**). The rapid equilibrium of antigen/antibody reaction implied that the Mab we developed may have a high association rate constant and dissociation rate constant (*41*). Considering the rapidity of manipulation, incubation time of antigen and antibody were chosen at 15 min. Compared with more than 90 min for previous ELISAs developed (*10, 13–15, 25, 26*), the analysis time of the present ELISA was reduced to 40 min by shortening the antigen/antibody incubation time. With this tremendous saving in time, this rapid ELISA provided higher analysis efficiency for the determination of Kan and Tob than conventional ELISA.

**Table 2.** Effects of Various Factors on Assay Performance of cdELISA ($n = 3$)

| | cdELISA for Kan | | cdELISA for Tob | |
|---|---|---|---|---|
| factor | $B_0$ | $IC_{50}$ (ng/mL) | $B_0$ | $IC_{50}$ (ng/mL) |
| | | pH value | | |
| 5.0 | 1.69 | 1.01 | 1.65 | 1.10 |
| 6.0 | 2.01 | 0.86 | 1.96 | 0.90 |
| 7.0 | 2.03 | 0.85 | 2.04 | 0.91 |
| 8.0 | 1.96 | 0.94 | 2.01 | 0.95 |
| 9.0 | 1.75 | 1.23 | 1.76 | 1.29 |
| | | ionic strength | | |
| 0.025 M PB | 1.97 | 0.87 | 2.01 | 0.85 |
| 0.05 M PB | 1.75 | 0.88 | 1.77 | 0.92 |
| 0.1 M PB | 1.46 | 0.95 | 1.51 | 1.01 |
| 0.2 M PB | 1.04 | 1.16 | 1.08 | 1.11 |
| 0.4 M PB | 0.83 | 2.47 | 0.91 | 2.63 |
| | | coating method | | |
| 4 °C, overnight | 1.41 | 0.82 | 1.45 | 0.85 |
| 37 °C, 2 h | 1.89 | 0.84 | 1.85 | 0.89 |
| | | coating buffer | | |
| 0.01 M PBS, pH 7.4 | 1.85 | 0.86 | 1.86 | 0.85 |
| 0.05 M CB, pH 9.6 | 1.34 | 0.91 | 1.41 | 0.92 |
| | | incubation time | | |
| 15 min | 1.89 | 0.83 | 1.87 | 0.86 |
| 30 min | 1.86 | 0.87 | 1.90 | 0.91 |
| 45 min | 1.91 | 0.89 | 1.86 | 0.85 |
| 60 min | 1.83 | 0.86 | 1.82 | 0.88 |

Based on these results, the standard curves of cdELISA for Kan and Tob were drawn as seen in **Figure 4**.

**Optimization of Colloidal Gold Immunoassay.** In general, the optimum pH value for antibody absorption on the gold surface was at the isoelectric point (p*I*) of the antibody molecule or 0.5 pH units higher. At this pH value, the antibody can maximally adsorb on the gold surface (*42*). The optimal pH and maximal antibody amount for conjugation can be determined by measuring the differential absorbance according to the method described by Paek et al. (*42*) and utilized by Sun et al. (*19*), Cho et al. (*20*), and Shim et al. (*24*). Using this method, we found that the optimal pH value for antibody absorption was 9.0, and the maximal antibody amount was 15 $\mu$g for 1 mL of colloidal gold at this pH value. In these conjugation conditions, the formed gold−antibody could be specifically captured by the coating antigen immobilized on the nitrocel-

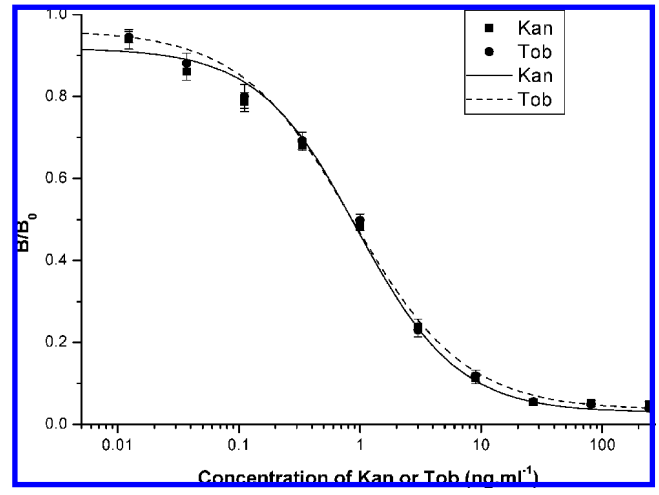


**Figure 4.** Standard curves of cdELISA for Kan and Tob. Data represent means and standard deviations of five determinations.

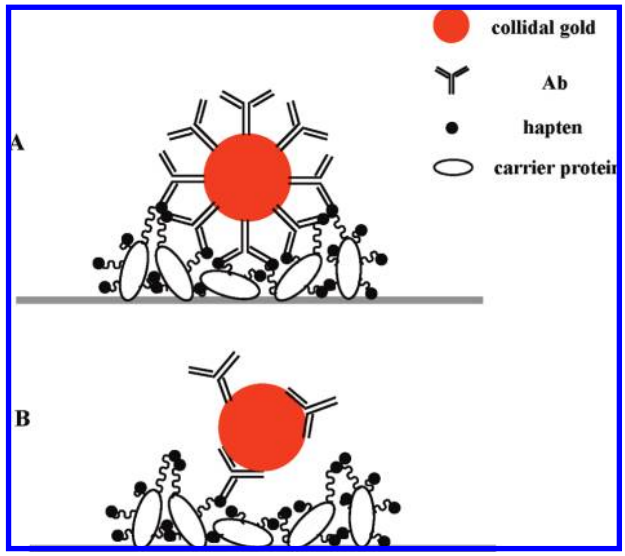


**Figure 5.** Schematic representation of interactions between colloidal gold-labeled antibodies and coating antigen. (**A**) Supposed model of colloidal gold−antibody with high avidity. (**B**) Supposed model of colloidal gold−antibody with low avidity.

lulose membrane. The combination, however, could not be replaced by Kan or Tob, even at high concentration (1 mg/mL). This result indicated that maximal antibody binding onto the gold surface may be harmful to the sensitivity of the strip test. Accordingly, to obtain the best sensitivity of the strip test, the pH values (6.0, 7.0, 8.0, and 9.0) and antibody amounts (2.5, 5, 10, and 20 $\mu$g per mL of colloidal gold) were screened in a two-dimensional titration for the optimum combination. As a result of titration, the optimal pH value was found to be 7.0 and the optimum amount of antibody was 5 $\mu$g for 1 mL of colloidal gold. In these conditions, the binding of colloidal gold-labeled antibody with coating antigen could be completely replaced by 5 ng/mL of Kan or Tob.

As the association constant of a multivalent antibody ($K_a$) can be expressed using the association constant of each combining site ($K_1$) as shown as

$$K_a = (K_1)^n$$

where $n$ is the number of combining sites (*43*), the avidity of a multivalent antibody could be greatly larger than the intrinsic affinity of its subunits (IgG), and the interaction of a multivalent antibody with a multivalent ligand could sometimes be irreversible (*44*). In addition, the steric hindrance could also affect the antibody/antigen combination and thus lead to the avidity variance of a multivalent antibody (*43, 45*). Therefore, as shown in **Figure 5**, it was assumed that excessive antibodies were conjugated to colloidal gold particles in the case of 15 $\mu$g antibody conjugation, and meanwhile, the orientation of the antibodies conjugated on the gold surface at pH 9.0 may be suitable for antibody/antigen interaction. Consequently, the avidity of the colloidal gold-labeled antibody (similar to IgM) was exceedingly large so that its binding with coating antigen was irreversible and could not be replaced by free hapten. In the case of 5 $\mu$g antibodies at pH 7.0 for conjugation, however, fewer antibodies bond on the gold surface, and the orientation of antibodies on the gold surface may hinder the antibody/antigen interaction. Thus, the avidity of gold−antibody decreased largely, and the best sensitivity of the strip test was obtained.

The size of colloidal gold particles is inversely proportional to the amount of trisodium citrate added in its preparation process (*22*). To evaluate the effect of the size of colloidal gold on the sensitivity of the strip test, several types of colloidal gold particles were prepared by adding 1, 1.6, or 2 mL of trisodium citrate into 100 mL of 0.01% chlorauric acid (m/v), which corresponded to diameter sizes of 40, 25, and 15 nm, respectively (*22*). As a result, it was found that the size of the colloidal gold particle has no significant effect on assay sensitivity. As we observed in the study that the smaller size of the gold particle has better dispersibility in solution than larger gold particles after conjugation with antibody, 15 nm of colloidal gold was used for further experiment.

Subsequently, some other physical-chemical factors affecting strip test performance were also evaluated. First, CB, PB, and PBS were tested as coating buffers, respectively. The better result was obtained with CB which resulted in a deeper color in the test line than PB and PBS. The effect of surfactant (Tween 20 and Triton 100) on assay performance was then evaluated. The existence of surfactant in the detection reagent can ensure the complete release of gold−antibody from the conjugate pad. By comparison, Triton 100 was prior to Tween 20 because the former showed higher staining intensity of the test line. As lower concentration of Triton 100 could not release the detector reagent

**Table 3.** Comparison of Results Obtained by the Strip Test and cdELISA

| | | | ELISA ($n$ = 5) | | | |
|---|---|---|---|---|---|---|
| | spiked level of Kan or Tob | strip test[a] | level found ± SD ($\mu$g/kg) | | recovery (CV) | |
| matrix | ($\mu$g/kg) | ($n$ = 5) | Kan | Tob | Kan | Tob |
| Meat | 0 | − − − − − | 1.1 ± 0.2 | 1.1 ± 0.2 | | |
| | 25 | ± ± ± ± ± | 15.8 ± 1.5 | 16.8 ± 1.7 | 63% (9.5%) | 67% (10.2%) |
| | 50 | ± + + + + | 35.5 ± 4.0 | 34.5 ± 3.0 | 71% (11.1%) | 69% (8.8%) |
| | 100 | + + + + + | 85.0 ± 12.0 | 81.1 ± 5.4 | 85% (14.1%) | 81% (6.7%) |
| | 200 | + + + + + | 192 ± 7 | 172 ± 14 | 96% (3.6%) | 86% (7.9%) |
| Liver | 0 | − − − − − | 0.6 ± 0.1 | 0.6 ± 0.1 | | |
| | 25 | ± ± ± ± ± | 18.3 ± 2.5 | 16.5 ± 1.2 | 73% (13.6%) | 66% (7.4%) |
| | 50 | ± + + + + | 35.3 ± 5.0 | 36.5 ± 3.7 | 71% (14.2%) | 73% (10.2%) |
| | 100 | + + + + + | 76.9 ± 7.8 | 70.2 ± 8.6 | 77% (10.2%) | 70% (12.3%) |
| | 200 | + + + + + | 164 ± 15 | 150 ± 17 | 82% (8.8%) | 75% (11.1%) |
| Kidney | 0 | − − − − − | 1.2 ± 0.2 | 1.2 ± 0.2 | | |
| | 25 | − − − − − | 13.0 ± 2.0 | 15.8 ± 2.1 | 52% (15.3%) | 63% (13.6%) |
| | 50 | ± ± ± ± + | 31.2 ± 4.1 | 30.5 ± 4.0 | 62% (12.8%) | 61% (13.2%) |
| | 100 | + + + + + | 73.4 ± 4.7 | 75.1 ± 9.4 | 73% (6.1%) | 75% (12.5%) |
| | 200 | + + + + + | 163 ± 13 | 154 ± 16 | 81% (8.1%) | 77% (10.3%) |

[a] −, absence of Kan and Tob; +, presence of Kan or Tob.

from the conjugate pad completely and higher concentration of surfactant may interfere with antibody/antigen reaction, 0.5% (v/v) Triton 100 was finally selected. Lastly, several types of nitrocellulose membrane (AE99, AE100, FF85, and Prima85, Whatman) were screened. All the membranes, except for AE100 which gave lower sensitivity, showed similar sensitivity. However, the highest staining intensity and best sharpness of the line were obtained with FF85, thus it was finally chosen.

The lowest detection limit is defined here as the concentration of Kan or Tob in the sample solution that just caused total invisibility of the test line. Under the optimal conditions described above, the detection limit of this one-step strip was measured using different concentrations of Kan or Tob in PBS. Although 2.5 ng/mL of Kan or Tob gave a considerable decrease in the assay signal, the limit of detection was set as 5 ng/mL in PBS to maximally avoid a false negative result.

**Analysis of Spiked Samples.** Previous studies conducted by Kitagawa et al. (*10*), Watanabe et al. (*12*), Loomans et al. (*14*), and Jin et al. (*15*) have demonstrated the development of ELISA or immunochromatographic assay for determination of Kan in serum and milk. However, the detection of Kan or Tob in a more complex matrix such as animal tissues has not been reported. Herein, a rapid ELISA and a strip test for Kan and Tob in swine tissues were performed. For cdELISA, trichloroacetic acid solution was used to deproteinate and extract Kan and Tob from tissues. The extract was then diluted with the optimized assay buffer. The matrix effect on ELISA performance in different dilution folds was investigated. As the absorbance at each concentration in 50-fold diluted extraction solution was almost the same as that in the assay buffer, 50-fold dilution was sufficient to eliminate the matrix effect on assay performance. For the strip test, neutral PBS was used as extraction solution. For detection on site, no centrifuge step was needed, and the sample extracts were directly pipetted onto the sample pad for analysis.

Samples with different spiked concentrations of 0, 25, 50, 100, and 200 $\mu$g/kg were simultaneously analyzed by the strip test and cdELISA described above. The results are shown in **Table 3**. For ELISA, recoveries of Kan and Tob in meat ranged from 63 to 96% with coefficient variations (CVs) of 3.6–14.4%, in liver ranged from 66 to 82% with CVs of 7.4–14.0%, and in kidney ranged from 52 to 81% with CVs of 6.1–15.3%. The detection limits, which were defined as the mean background level plus 3 times the standard deviation (SD), were 1.7 $\mu$g/kg in meat, 0.9 $\mu$g/kg in liver, and 1.8 $\mu$g/kg in kidney, respectively. For the strip test, the detection limits were estimated to be 50 $\mu$g/kg in meat and liver and 100 $\mu$g/kg in kidney, respectively.

We reported here the development and optimization of a rapid ELISA and a strip test for Kan and Tob in swine tissues using an anti-Kan Mab. The immunoassays can be used to sift large numbers of samples for potential noncompliant results (*46*). Whether the suspected noncompliant samples contain Kan or Tob could be further confirmed by confirmatory methods such as that of Kaufmann, A. et al. (*6*) and USDA (*7*).

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**Received for review December 12, 2007. Revised manuscript received March 5, 2008. Accepted March 7, 2008. This work is supported by the National Key Technologies Research and Development Program of China during the 11th Five-Year Plan Period (No.2006BAK10B090).**

JF703602B