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Immunoassay

APPLICATION OF SUSPENSION ARRAY FOR SIMULTANEOUS DETECTION OF ANTIBIOTIC RESIDUES IN RAW MILK

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Suspension array technology enjoys great potential in simultaneous detection of drug residues in the field of food safety. A monoclonal antibody against gentamycin was produced and a bead-based indirect competitive fluorescent immunoassay was developed using the suspension array system for the simultaneous detection of antibiotic residues such as gentamycin and kanamycin in raw milk. The limit of detection (LOD) for kanamycin and gentamycin were 0.5 ng/mL and 4.1 ng/mL in a phosphate-buffered saline (PBS), 2.2 ng/mL and 12.2 ng/mL in milk. The monoclonal antibodies used for the multiplexed assays showed no significant cross-reactivity with other aminoglycoside antibiotics. To validate the method, milk samples spiked with gentamycin or kanamycin were detected using suspension array technology and conventional enzyme-linked immunosorbent assay (ELISA) in parallel. The good correlation between the obtained results showed that the method is feasible in actual application, and it also laid a firm foundation for establishing a multi-target assay for more actual application.

Keywords: Antibiotic residues; Food safety; Multi-targets detection; Suspension array

INTRODUCTION

Suspension array system is a flow-based dual-laser system for simultaneous identification and quantification of up to 100 different analytes in a single biomolecular assay. It is a flexible technical platform based on Flexible Multi-Analyte

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Profiling (xMAP) technology which was developed in recent years (Morgan et al. 2004). It combines encoded microspheres with flow cytometry. There are two lasers to analyze the microspheres in a flow stream. The first laser indentifies each microsphere associated analyte according to the fluorescent signature of the microsphere, and the second measures the reporter molecules attached to the analytes that quantifies the amount of the analyte (Nolan and Sklar 2002). Many studies have been carried out in the field of clinical diagnosis and genomic research by combining flow-based technology with immunoassays (Yan et al. 2004; Fuja, Hou, and Bryant 2004). Beads of different sizes or colors or a single bead with different fluorescent probes are used for multiplexed immunoassay. Previous studies using multiplexed assay technology including detecting thyroxine and thyrotropin from blood-spot sample for congenital hypothyroidism (Bellisario, Colinas, and Pass 2000), antibodies to West Nile virus in human serum and cerebrospinal fluid (Wong et al. 2004); Ig classes in serum and stool samples (Dasso et al. 2002), identifying response patterns in hyperinflammatory diseases (Hsu et al. 2008); and cytokines in human serum (Ray et al. 2005). As an open platform, xMAP was gradually introduced into detecting and quantifying many virus and drug residues in food and agriculture samples. For example, a multiplex microsphere immunoassay was developed to monitor seed potatoes for potato virus X, Y and PLRV (Bergervoet et al. 2008), and a flow cytometric immunoassay was developed for the detection of residues of sulfonamides in milk (de Keizer et al. 2008).

However, at present, the multiplexed immunoassays using suspension array technology are more prevalent for analyzing viruses and antibodies than detecting small molecular weight compounds such as drug residues. Popular techniques for drug residue detection are high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), and enzyme-linked immunosorbent assay (ELISA) (Gentili 2007; Jin et al. 2005). The chromatography-based methods are laborious and expensive and more suitable for confirmation. The conventional immunoassay methods are simple and rapid for screening but limited by its single-target detection. A simple, rapid, and multi-target screening method is required for drug residue detection. Recent studies have reported on the suspension array system for the detection of drug residues for its simplicity, low cost, specificity, and multiplexing capabilities (Zou et al. 2008). Different problems were also pointed out, such as lack of application in detection of real samples (Liu et al. 2009) or weak fluorescent signals when detecting real samples (de Keizer et al. 2008).

In the present study, we applied suspension array technology to develop a multiplexed indirect competitive immunoassay for simultaneous detection of antibiotic residues, such as kanamycin and gentamycin, in raw milk. First, we produced a monoclonal antibody (McAb) against gentamycin successfully. Then, with the coating antigen of kanamycin and gentamycin been attached to different encoded beads and R-phycoerythrin-conjugated goat anti-mouse IgG as the fluorescent probe, a bead-based indirect competitive fluorescent immunoassay using Bio-Plex^{TM} 200 suspension array system was developed. In order to develop actual application, raw milk samples spiked with kanamycin or gentamicin were detected using ELISA and Bio-Plex^{TM} assays for comparison.

MATERIALS AND METHODS

Materials

Gentamycin sulfate, kanamycin sulfate, bovine serum albumin (BSA), Keyhole limpet hemocyanin (KLH), Freund's incomplete adjuvant, Freund's complete adjuvant, Dulbecco's Modified Eagle Medium (DMEM), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and sulfo N-hydroxysulfosuccinamide (sulfo-NHS) were purchased from Sigma Chemical Co. (St. Louis, MO). The McAb against kanamycin was a gift from the Chinese Academy of Inspection and Quarantine (Beijing, China). The R-phycoerythrin(R-PE)-conjugated goat anti-mouse IgG obtained for Abcam Inc. (Cambrige, UK) was used as the biological probes. The BALB/C mice were purchased from the Chinese Academy of Military Science Laboratory Animal Center (Beijing, China), and the ultrapure water used for the experiments was generated by a Milli-Q system from Millipore (Bedford, MA).

Instrumentation

The Bio-Plex^{TM} 200 system, which was used for evaluating the multiplexed assays and Bio-Plex^{TM} COOH beads (numbers 20, 30), were purchased from Bio-Rad Laboratories Inc. (Hercules, CA). MultiScreen Filter 96-well Plates were from Millipore (Bedford, MA). Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) AB4700 that was employed for molecular weights determination was purchased from Applied Biosystems (Carlsbad, CA). High-binding polystyrene 96-well plates were from Corning (New York, UK). The microplate reader for ELISA was from Tecan Inc. (Hombrechticon, Switzerland).

Preparation of Artificial Antigen

Gentamycin-KLH and gentamycin-BSA conjugates were coupled as immunogen and coating antigen separately using an ester activation method (Haasnoot et al. 1999). In brief, a reaction mixture containing 45 mg gentamycin and 10 mg protein (BSA or KLH) was dissolved in 1.5 mL PBS (0.01 M, pH 7.4) and aqueous EDC solution (0.3 mg/L) was added drop by drop to the mixture. After stirring at room temperature for 60 min and letting stand at 4°C overnight, the mixture was subject to dialysis against PBS at 4°C for 3 days and then stored at −20°C until use. The procedure to couple kanamycin-BSA conjugate was completed with the same process as the gentamycin-BSA.

Mcab Production

The McAb against gentamycin was produced as described previously (Harlow and Lane 1988) with some modification. In brief, the eight-week-old BALB/C female mice were immunized with immunogen and a like amount of Freund's complete adjuvant by intraperitoneal injection at a dosage of 100 μg/mouse. Then, a booster dose of Freund's incomplete adjuvant was given to the mice (100 μg/mouse)

3 times with an interval of two weeks. Ten days after each injection, serum was collected and the titer was monitored by ELISA. The splenocytes from the mouse with serum showing highest titer were fused with SP2/0 myeloma cells and cultured in 96-well plates. Ten days after cell fusion, the hybridoma cells were screened by an indirect competitive ELISA and subcloned with the limiting dilution method. At last, the selected hybridoma cells were injected intraperitoneally into eight-week-old BALB/C female mice to produce McAb in the ascites fluid. And, the purification of the ascites fluid was carried out using protein G Sepharose™ chromatography.

Protein Beads Conjugation

The carboxylated polystyrene beads (numbers 20, 30) were coupled with kanamycin-BSA and gentamixin-BSA conjugates via carbodiimide reaction involving the protein primary amino groups and the carboxyl functional groups bound on the surface of beads (Hermanson 2008). In general, 100 μL beads (1.25×10^7 beads/mL) were suspended and washed. The pelleted beads were activated with EDC and NHS for 20 min at room temperature. Then, the mixture of activated beads and coating antigen was vortexed for 2 h at room temperature. After that, the coated beads were blocked with blocking buffer (1% BSA in PBS) for 30 min at room temperature. At last, the beads were resuspended in 150 μL of PBS and stored at 4°C. The optimal amounts of kanamycin-BSA and gentamycin-BSA conjugates for coupling were determined by coating beads with different concentrations of coating antigen. Coupling controls were examined by adding sufficient detection antibodies and staining 5000 of each type of beads with R-phycoerythrin-conjugated goat anti-mouse IgG. An amount of 100 beads of each type were read out and corresponding median fluorescent intensities (MFIs) were obtained by Bio-Plex™ 200 suspension array system.

Bio-Plex™ Multiplexed Assay

Standard curves. The assay was developed in an indirect competitive immunoassay format. Different detection McAb concentrations need to be first optimized by varying amounts of anti-kanamycin McAb and anti-gentamycin McAb, respectively, in the 100-μL assays with fixed numbers of coating antigen-coated beads (5000 beads/well). Then, in brief, the MultiScreen 96-well filter plate was prewetted with 150 μL PBS per well. The 5000 coating antigen-coated beads were used in each assay. To prepare a standard curve, standard solutions of kanamycin and gentamycin were added to PBS at concentrations of 1000, 100, 10, 5, 1, 0.5, 0.1, and 0 ng/mL or to negative milk samples at concentrations of 9000, 4500, 900, 90, 9, 0.9, 0.09, and 0 ng/mL. Amounts of 50 μL of standard solutions were added to each well plus 50 μL monoclonal antibody solution in triplicate. This mixture was incubated in the dark for 1 h at 37°C. Subsequently, PE-conjugated goat anti-mouse IgG (2.5 μg/mL) was added to the plate after being vacuumed. This mixture was incubated in the dark for another 1 h at 37°C. At last, the filter plate was vacuumed and the beads were resuspended with 125 μL PBS for detection by Bio-Plex 200 system.

Cross-reactivity. The cross reactivity of aminoglycosides were assessed with both of the detection antibodies using the Bio-Plex™ assay. Aminoglycosides, like kanamycin, gentamycin, amikacin, apramycin, tobramycin, and sisomicin, were added to PBS at concentrations of 100000, 10000, 1000, 100, 10, 1, 0.1, and 0 ng/mL, respectively, and tested using an indirect competitive immunoassay. The cross reactivity values were calculated as described previously (Shen et al. 2007).

Recovery. Raw milk was spiked with kanamycin and gentamycin with final concentrations of 10, 25, and 50 ng/mL. The recovery experiments were performed in triplicate at least 3 times on different days and the recoveries of kanamycin and gentamycin from the spiked milk were calculated on the basis of the standard curves constructed by Bio-Plex™ assays.

Elisa

The assay is based on indirect competitive reaction theory, too. High-binding polystyrene 96-well plates were coated overnight at 4°C with the same coating antigens used in the Bio-Plex™ assay. For kanamycin and gentamycin, assays, plates were blocked for 2 h at 37°C with blocking buffer (1% BSA in PBS), then washed three times. For analysis, 50 μL of standards or unknowns and 50 μL monoclonal antibody solutions were added in triplicate, incubated for 1 h at 37°C, followed by three plate washings. An amount of 100 μL/well anti-mounse IgG – alkaline phosphatase (0.5 ng/mL before dilution) was added; the plate was then incubated for 1 h at 37°C. With final washings performed, the plate was incubated for 30 min with substrate reagent and read at 405 nm with a microplate reader.

Preparation of Standard Samples

All samples were defatted by centrifugation 10 min at 12000 × g after being diluted 10 times in PBS prior to measurement. The milk samples served as negative control samples or for preparation were taken from individual cows with good health and at least 8 weeks after any treatment with antimicrobial substances according to Xie et al. (2009).

RESULTS AND DISCUSSION

Verification of Coating Antigen by MALDI-TOF-MS

Because hapten, similar to gentamycin and kanamycin, has single antigenic determinants, BSA was selected as the carrier protein. Determined by MALDI-TOF-MS, the molecular weight (MW) of BSA, kanamycin-BSA, and gentamycin-BSA conjugate are 66438Da, 71795Da, and 72924Da, respectively, as showed in Figure 1, and the standard molecular weights of kanamycin and gentamycin are 485Da and 477Da, respectively. The coupling ratio of coating antigen was calculated with the following equation:

$$\text{Coupling ratio} = (\text{MW}_{\text{hapten-BSA}} - \text{MW}_{\text{BSA}})/\text{MW}_{\text{hapten}}$$

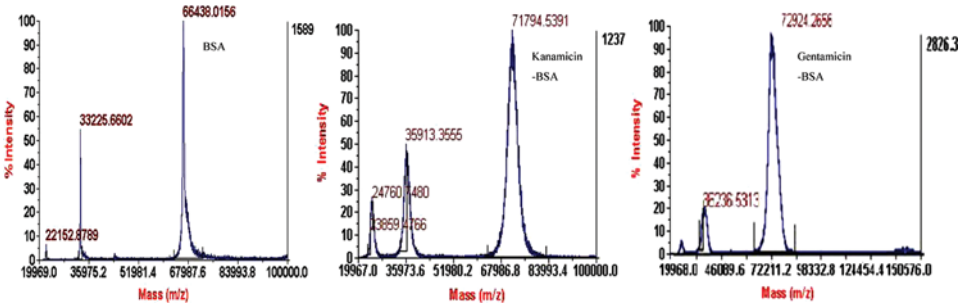

Figure 1. m/z of kanamycin-BSA, gentamycin-BSA conjugates, and BSA determined with MALDI-TOF. (Figure available in color online.)

Therefore, the coupling ratios of kanamycin and gentamycin were 11:1 and 14:1, respectively, and the coupling ratio proved a successful conjugation between hapten and BSA.

Production of McAb

The hybridoma named 4H12 was chosen as the most sensitive and specific one for gentamycin, and the McAb was obtained from ascites of BALB/C mice. The titer of the McAb was $1{:}1 \times 10^5$ measured by ELISA.

Protein Beads Conjugation

To ensure intra-assay precision between different batches of protein-coated beads, it was necessary to determine the optimal concentration of coating for coupling the conjugates to beads repeatedly and steadily in the long term. The optimal amounts of kanamycin-BSA and gentamycin-BSA conjugates for coupling were determined by coating beads with different concentrations of coating antigen. As shown in Figure 2(a), MFIs increased with the addition of conjugates and tended to be plain after reaching a certain climax; 8 μg of kanamycin-BSA and 6 μg of gentamycin-BSA were considered the optimal amounts for coupling with 100 μL of each type of beads (1.25×10^7 beads/mL) as a batch.

Standard Curves

The assay was developed in an indirect competitive immunoassay format. The amount of detection McAb against kanamycin and gentamycin were first optimized. As shown in Figure 2(b), the optimal amounts were approximately 25 ng anti-kanamycin McAb and 50 ng anti-gentamycin McAb per 5000 beads based on the MFIs.

Using Origin 7.5 (Microsoft, Redmond, Washington) with logistic fitting function for regression analysis, the standard curves both in PBS and in raw milk extracts were constructed and presented in Fig. 3, in which MFI_0 represents the MFI obtained from blank sample. The MFIs obtained with these samples were compared

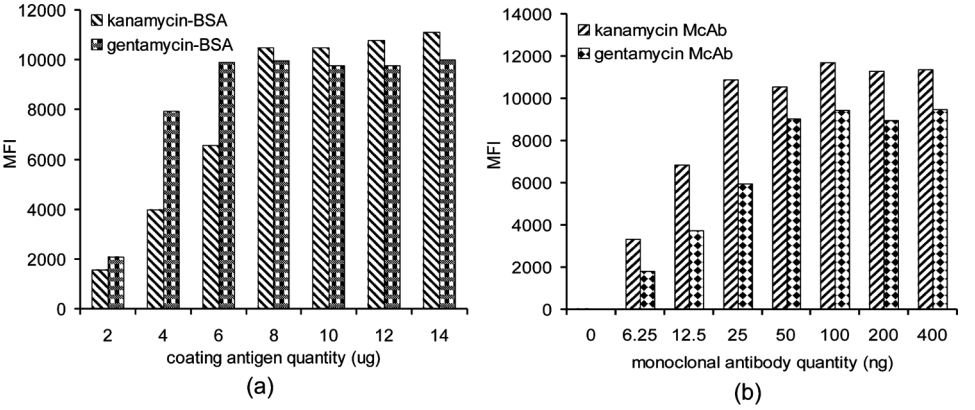

Figure 2. Optimization of amounts of coating antigen (a) and detection antibodies (b).

with the MFI_0 to determine the percentage of inhibition. The MFI_0 shown in Table 1 indicates that the survival lipids and protein in the milk extracts affect the fluorescent signals a little. The LOD for kanamycin and gentamycin were 0.5 ng/mL and 4.1 ng/mL in PBS and 2.2 ng/mL and 12.2 ng/mL in milk. Details are shown in Table 1, in which IC_{50} represents the 50% of inhibition value.

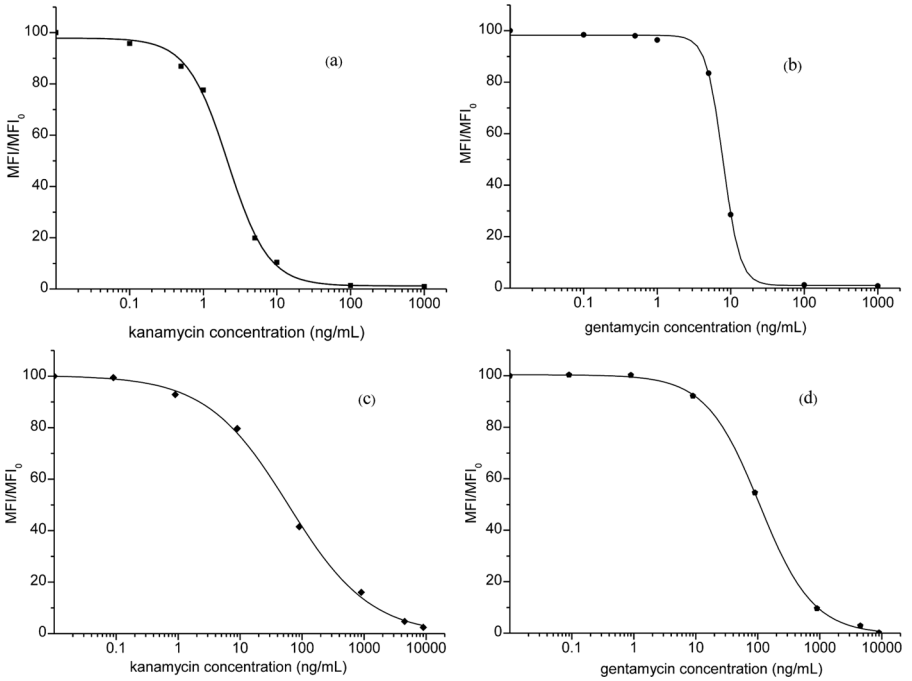

Figure 3. Standard curves of kanamycin and gentamycin in PBS and in milk extract. (a) and (b) were obtained simultaneously in PBS; (c) and (d) were obtained simultaneously in milk extract.

Table 1. The sensitivities of kanamycin and gentamycin in different matrix obtained by Bio-Plex™ multiplexed assay

		In PBS	In milk extracts
Kanamycin	MFI_0	10848.3	6800.5
	LOD (ng/mL)	0.5	2.2
	IC_{50} (ng/mL)	2.3	59.1
Gentamycin	MFI_0	9464.3	7592.5
	LOD (ng/mL)	4.1	12.2
	IC_{50} (ng/mL)	7.1	107.2

Cross Reactivity

The cross reactivity values were calculated with the following equation:

$$\text{Cross reactivity } (\%) = IC_{50} \text{ (kanamycin or gentamycin)}/IC_{50} \text{ (aminoglycoside)} \times 100\%$$

The results shown in Table 2 indicate that the McAb exhibited no significant alteration or cross-reaction with other aminoglycoside antibiotics, which also implies the good specificity of the anti-gentamycin McAb and anti-kanamycin McAb.

Comparison of Recoveries Obtained by Bio-Plex™ Assay and ELISA

By adding known quantities of the stock solution of kanamycin and gentamycin into negative raw milk at three different levels (15 ng/mL, 50 ng/mL, and 100 ng/mL) within the desired working range, recoveries of the multiplexed assay were calculated and the results are summarized in Table 3. It has been approved by the European Community that the maximum residue limit (MRL) of gentamycin in cow milk is 100 μg/kg and the MRL of kanamycin has been set to 150 μg/kg for milk. Table 3 shows a comparison of the detection results obtained by the Bio-Plex assays and ELISA. The analysis shows that there is good correlation between the results of this assay and those of ELISA.

Table 2. Cross reactivity of aminoglycoside antibiotics with the McAb of gentamycin and kanamycin in PBS

	Cross-reactivity (%)	
Analogues	Kanamycin	Gentamycin
Gentamycin	<0.01	100
Kanamycin	100	<0.01
Amikacin	<0.01	<0.01
Apramycin	<0.01	<0.01
Tobramycin	<0.01	<0.01

Table 3. Comparison of the recovery results obtained by Bio-Plex™ multiplexed assay and ELISA

		Bio-Plex multiplexed assay		ELISA	
Sample	Spiked level (ng/mL)	Recovery (%) ± SD	CV (%)	Recovery (%) ± SD	CV (%)
Kanamycin	15	109 ± 83	1.72	107 ± 0.06	6.37
	50	95 ± 97	2.70	97 ± 0.1	11.99
	100	87 ± 296	10.47	94 ± 0.02	3.11
Gentamycin	15	90 ± 497	7.47	90 ± 0.2	11.01
	50	99 ± 634	13.74	110 ± 0.05	5.09
	100	120 ± 78	2.26	106 ± 0.06	9.01

CONCLUSIONS

A McAb against gentamycin was obtained successfully and a bead-based indirect competitive fluorescent immunoassay was developed for rapid detection of small molecule drug residues in animal-derived food products, such as raw milk. Especially, even with the matrix affection of milk, the MFIs detected from raw milk samples are still strong enough and sufficient to detect the lowest allowable quantity of the antibiotics in milk at MRL level. In fact, cephalosporins and sulfonamides, which are used worldwide in cows and are also tested in milk, could be added to the assay as the suspension array technology allowing up to 100 different combinations of assays. Additionally, compared with ELISA, the multiplexed assay had a similar LOD, but its ability to measure several analytes simultaneously in a single sample is superior to ELISA. As an easy, effective, time-saving method, the bead-based immunoassay has the potential to advance the detection of drug residues in actual application. Additionally, our research laid a firm foundation for simultaneous screening of multiple analytes in more food products. Further research could involve adding more drug residues to the assay and establishing a reliable multiplexed assay for detecting analytes in more real samples, such as tissue, fat, among others.

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