Research Article

Analysis of the Kanamycin in Raw Milk Using the Suspension Array

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With the monoclonal antibody against kanamycin being prepared successfully, a bead-based indirect competitive fluorescent immunoassay was developed to detect kanamycin in milk. The fact that there was no significant cross-reaction with other aminoglycoside antibiotics implied that the monoclonal antibody was highly specific for kanamycin. The limit of detection (LOD) and the 50% inhibition concentration (IC50) in raw milk were 3.2 ng/mL and 52.5 ng/mL, respectively. Using the method developed in this study, the kanamycin concentrations were monitored in raw milk after the intramuscular administration of kanamycin in sick cows. Compared to the conventional enzyme-linked immunosorbent assay (ELISA), the method using the suspension array system was more sensitive. The results obtained in the present study showed a good correlation with that of the ELISA.

1. Introduction

Kanamycin is an aminoglycosides antibiotic widely used in veterinary medicine for the treatment and the prevention of microbial infections. If the withdrawal periods were disobeyed after the administration, the milk from the medicated animals might contain kanamycin residues. The European Union has established provisional maximum residue limits (MRLs) of 0.15 ppm for milk [1].

In order to monitor the kanamycin residue levels in livestock products, simple and reliable analytical methods are required. So far, various detection methods of kanamycin residues have been used, including the high-performance liquid chromatography [2], the gas chromatography-mass spectrometry [3], microbioassay [4], and the ELISA [5, 6]. As the chromatography-based methods being time-consuming and expensive, immunoassays like the ELISA become relatively faster and cheaper. Nowadays, suspension array system, which is a flow-based dual-laser system for simultaneously identifying and quantitating up to 100 different analytes in a single biomolecular assay, was gradually introduced in the detection of drug residues as a screening method in the field of food safety. Liu et al. once developed a simultaneous detection model for three kinds of veterinary drugs by the suspension array technology. However the method did not consider the application in real samples containing a complex matrix [7].

In this study, we first prepared a monoclonal antibody against kanamycin. Secondly, with the coating antigen of kanamycin being attached to the encoded beads and the R-phycoerythrin-conjugated goat anti-mouse IgG as the fluorescent probe, a bead-based indirect competitive immunoassay was developed by the suspension array technology. The approach was based on the fluorescent detection using the Bio-Plex 200 system (BioRad, USA). With a particular focus on the application in real samples containing a complex matrix, kanamycin was administered intraperitoneally in the cows at a dose of 10 ng/kg (twice per day for 3 days). The milk samples were taken and detected by the bead-based indirect competitive immunoassay and the ELISA separately to monitor the kanamycin residues in raw milk.

2. Materials and Methods

2.1. Materials. The kanamycin sulfate, the bovine serum albumin (BSA), the keyhole limpet hemocyanin (KLH), the
Freund incomplete adjuvant, the Freund complete adjuvant, the Dulbecco modified Eagles medium (DMEM), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and the sulfo N-hydroxysulfo succinimide (sulfo-NHS) were purchased from the Sigma Chemical Co. (USA). The BALB/C mice were purchased from the Chinese Academy of Military Science Laboratory Animal Center (China). The R-phycocerythrin-(R-PE-) conjugated goat anti-mouse IgG obtained for the Abcam Inc. (UK) was used as biological probes. The ultrapure water used for the experiments was generated by a Milli-Q system from Millipore (USA).

**2. Results**

3.1. ELISA. The titer of the McAb was $1:1 \times 10^5$ measured by the ELISA. The cross-reactivity of the purified monoclonal antibody was assessed by the addition of different concentrations of kanamycin, gentamycin, neomycin, streptomycin, apramycin, amikacin, apramycin, neomycin, and apramycin.

<table>
<thead>
<tr>
<th>Analogous compound</th>
<th>Cross-reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kanamycin</td>
<td>100</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Neomycin</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Amikacin</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Apramycin</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

**FIGURE 1:** The calibration curves in the raw milk extract constructed by competitive ELISA, in which $B$ values are measured using enzyme-labelled meter and $B_0$ represented $B$ value obtained from the blank samples.

2.2. The Preparation of the Monoclonal Antibody. Briefly, the entire procedure was composed by four fundamental steps: firstly, the kanamycin-KLH and the kanamycin-BSA conjugates were synthesized as the immunogen and the coating antigen separately using the easter activation method [8]; secondly, the mice were immunized; thirdly, the stable hybridoma cells were produced. And finally, the monoclonal antibody was prepared from the ascites fluid of the mice as described previously [9]. The purification of the ascites fluid was conducted using the protein G sepharose chromatography.

2.3. The Bio-Plex Assay Protocol. The Bio-Plex COOH beads (1.25 × 10^7 beads/mL, BioRad, USA) were coupled with the coating antigen according to the manufacturer’s protocol firstly. The assay was developed in an indirect competitive immunoassay format. The standard solutions were prepared at the concentrations of 1,000, 100, 50, 25, 10, 5, 2.5, 1, and 0 ng/mL. MultiScreen 96-well filtration plates (Millipore, USA) were used as reaction vessels. To prepare a standard curve, 50 µL of the standard solutions and 50 µL of the monoclonal antibody solution were added to each well plus 5,000 of the kanamycin-BSA-coated beads per well. The R-PE-conjugated goat anti-mouse IgG solutions (2.5 µg/mL) were added to each well followed by an incubation at 37°C for 1 hour. After another incubation at 37°C for 1 hour, the fluorescent signal binding to the beads was detected by the Bio-Plex 200 system. The serial dilution 1:2 of the McAb (from 4 µg/mL to 0.125 µg/mL) was used to determine the optimal working concentration of the McAb. The limit of detection (LOD) was determined to be 10% inhibition concentration.

2.4. ELISA. The assay was based on the indirect competitive reaction theory, too. Polystyrene 96-well plates were coated overnight at 4°C with the same coating antigens used in the Bio-Plex assay. The plates were then blocked with 3% nonfat dry milk for 2 hours at 37°C and washed three times with PBS/0.05% Tween 20. The same dilution used in the Bio-Plex assay was used for all standards, unknowns, and monoclonal antibody. The standards, the unknowns, and the monoclonal antibody solutions were added at 50 µL per well and incubated for 1 hour at 37°C. It was then washed immediately for three times. The appropriate anti-mouse IgG-alkaline phosphatase (0.5 ng/mL before the dilution) was added at 50 µL per well. It was then incubated for 1 hour at 37°C. With the final washings performed, the plates were incubated for 30 minutes with the substrate reagent and were read at 405 nm with a microplate reader.

2.5. The Monitoring of the Raw Milk Concentrations. The kanamycin was administered intraperitoneally in three cows at a dose of 10 mg/kg (twice per day for 3 days). The milk samples were collected 7, 14, 25, 37, 49, 61, 73, 85, 97, and 109 hours after the last injection of the kanamycin. All milk samples and spiked standard solutions were defatted by centrifugation for 10 minutes at 4°C after 10 times dilution in phosphate-buffered saline (PBS). The supernatants were subjected to the bead-based indirect competitive immunoassay and the ELISA separately to determine the kanamycin concentrations in the milk samples. All the samples and the standard solutions were run in triplicate.

**TABLE 1:** The cross-reactivities of kanamycin and other analogous compounds.
T2: Intra-and inter-assay validation of the microsphere-based fluorescence immunoassay.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>1</th>
<th>2.5</th>
<th>5</th>
<th>10</th>
<th>25</th>
<th>50</th>
<th>100</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFI/MFI₀ (%) (intra-assay)</td>
<td>96.9</td>
<td>91.5</td>
<td>86.1</td>
<td>78.6</td>
<td>64.5</td>
<td>51.8</td>
<td>38.4</td>
<td>11.9</td>
</tr>
<tr>
<td>SD (n = 3)</td>
<td>2.8</td>
<td>7.9</td>
<td>5.3</td>
<td>3.0</td>
<td>0.7</td>
<td>3.4</td>
<td>4.6</td>
<td>2.5</td>
</tr>
<tr>
<td>Intra-CV%</td>
<td>2.9</td>
<td>8.7</td>
<td>6.2</td>
<td>3.8</td>
<td>1.1</td>
<td>6.6</td>
<td>12.0</td>
<td>20.6</td>
</tr>
<tr>
<td>MFI/MFI₀ (%) (inter-assay)</td>
<td>96.3</td>
<td>92.3</td>
<td>86.9</td>
<td>76.8</td>
<td>62.0</td>
<td>52.4</td>
<td>37.8</td>
<td>12.2</td>
</tr>
<tr>
<td>SD (n = 3)</td>
<td>1.8</td>
<td>2.4</td>
<td>7.9</td>
<td>3.5</td>
<td>2.4</td>
<td>5.1</td>
<td>4.3</td>
<td>2.0</td>
</tr>
<tr>
<td>Inter-CV%</td>
<td>1.9</td>
<td>2.6</td>
<td>9.1</td>
<td>4.5</td>
<td>3.8</td>
<td>9.8</td>
<td>11.5</td>
<td>16.7</td>
</tr>
</tbody>
</table>

Table 2: Intra- and inter-assay validation of the microsphere-based fluorescence immunoassay.

![Figure 2](image-url)

**Figure 2:** Optimization of working concentration of the kanamycin monoclonal antibody according to the median fluorescent intensity (MFI) obtained by the Bio-Plex 200 system.

amikacin, and apramycin, ranged from 0 ng/mL to $10^5$ ng/mL in PBS. The results calculated in Table 1 indicated that the monoclonal antibody did not show any cross reactivity with other aminoglycosides.

A conventional ELISA was first performed for comparison, and the LOD for the kanamycin in milk was 5 ng/mL according to the calibration curve shown in Figure 1.

3.2. The Microsphere-Based Fluorescence Immunoassay. An appropriate working concentration of kanamycin monoclonal antibody was selected according to the median fluorescent intensity (MFI) obtained by the Bio-Plex 200 system (Figure 2).

The calibration curves in the raw milk samples were constructed and presented in Figure 3, in which the MFI₀ represented the MFI obtained from the blank sample. A logistic fitting function was applied to convert the MFI into concentration values. The determination coefficients $R^2$ was 0.997 in milk. The LOD and the IC₅₀ for the kanamycin in milk were 3.2 ng/mL and 52.5 ng/mL, respectively.

To evaluate the performance of the developed assay, standard deviation (SD) and CV% were analyzed (as shown in Table 2). Intra-assay precision is represented by intra-CV%, and inter-assay precision is alternatively indicated by inter-CV%. The intra-CV% was in the range of 1.1–20.6, and the inter-CV% was in the range of 1.9–16.7.

![Figure 3](image-url)

**Figure 3:** The calibration curves in the raw milk extract, in which MFI₀ represented the MFI obtained from the blank samples.

3.3. The Comparison of Two Methods in the Measurement of the Raw Milk Samples. Thirty raw milk samples were collected from three cows. Figure 4 showed a comparison of the detection results obtained by the bead-based competitive fluorescent immunoassay and the ELISA. The analysis showed that there was a good correlation between the results of the two assays with the correlation coefficient: $r = 0.978$ ($P > 0.05$). Basically, the kanamycin concentrations in raw milk decreased gradually. 7 hours after the last injection, the residues reached 190 ng/mL. 20 more hours later, the residues reduced to 40 ng/mL, which was lower than the MRL provided by the European Union.

4. Discussion

In the present study, a McAb against kanamycin was obtained successfully. A bead-based indirect competitive fluorescent immunoassay was developed for the rapid detection of small-molecule drug residues in animal-derived food products, for example, raw milk. Especially, with the matrix affection in milk, the MFI detected in the raw milk samples was still strong enough for the residue determination.

It indicated that the beads coupling can be performed. As an easy, effective, time-saving method, the bead-based immunoassay had the potential to gear up the detection of the drug residues in actual applications. It also laid...
a firm foundation for the simultaneous screening of multiple analytes in food products.

In fact, for a single analysis, the cost was close to that of the ELISA. However in case of multiplex detections, the cost could be reduced. The main aim of the present study was to apply the method in food analysis and to solve the problem in sample processing.

Acknowledgments

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References


