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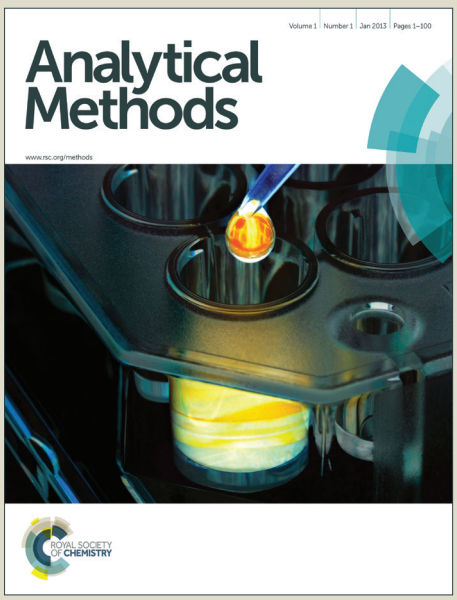

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Development of an enzyme-linked immunosorbent (ELISA) for natamycin residues in foods based on a specific monoclonal antibody

Yanni Chen, Dezhao Kong, Liqiang Liu, Shanshan Song, Hua Kuang, Chuanlai Xu*

An indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) was developed based on a sensitive and specific monoclonal antibody (mAb) against natamycin (Nata) for Nata detection in milk, juice, yoghurt, and cheese samples. The working range of ic-ELISA was 0.64–4.46 μg L^{-1} with an IC_{50} value of 1.69 μg L^{-1}. The average recoveries of milk, juice, yoghurt, and cheese samples spiked with Nata were 103–121%, 103–121%, 84–114%, and 89–108%, respectively. The results indicated that ic-ELISA can be effectively applied for Nata analysis in these food products.

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Introduction

Foods are prone to be contaminated by microorganisms, which negatively affect food quality and consumers acceptance. Preservatives are commonly added to foods to inhibit microbial growth, ensure safety, and lengthen shelf-life [1]. Natamycin (Nata), which is extensively used as a food preservative [2-4], is produced through the fermentation of streptomycetes. Therefore, Nata is a natural antifungal compounds, which exhibits broad spectrum activity against yeast and mould by binding to sterols, specifically ergosterol, to restrain fungal growth [5, 6]. Nata cannot inhibit bacterial growth, therefore it cannot affect the natural mature process of yogurt [2], cheese [7], ham [8], or dry sausage [9]. Several countries and organizations have set regulatory levels for Nata residue in foods. Through Annex III Directive 95/2/EC, European Union states that Nata can be used as an additive for the surface treatment of semi-hard, semi-soft cheeses and dry, cured sausages at a maximum level of 1 mg dm^{-2} with a depth no greater than 5 mm [10]. According to the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA), the acceptable daily intake (ADI) of Nata should be 0.3 mg kg^{-1} body weight [11]. However, an early clinical study has reported that Nata induces nausea, diarrhea, anorexia and other symptoms, when used for systemic mycoses. Additionally, the daily ingestion of Nata can weaken the immune system [11]. As a result, it is necessary to develop effective methods for the detection of Nata residues in foods.

Several analytical methods have been established for Nata analysis in foods, including high performance liquid chromatography coupled to diode-array detection (HPLC-DAD) [10], reserved-phase high performance liquid chromatography (RP-HPLC) [12], high-performance liquid chromatography coupled to high resolution mass spectrometry (HPLC-HRMS) [13], and ultrahigh-performance liquid chromatography

coupled to tandem mass spectrometry (UHPLC-TMS) [11]. All these methods are both sensitive and specific; however, they require laborious sample pre-treatments that are time-consuming and costly. Additionally, expensive instruments and highly qualified personnel are required to implement these methods.

The enzyme-linked immunosorbent assay (ELISA) is cost-effective, sensitive, selective, and simple for the analysis of various samples including large analytes such as microorganism [14, 15] and proteins [16, 17] and small analytes such as heavy metals [18, 19], hormones [20, 21], pesticides [22, 23], and antibiotics [24-26]. To the best of our knowledge, a mAb -based ELISA for Nata detection in foods has not been developed. This paper aims to produce a specific and sensitive mAb and establish an indirect competitive ELISA (ic-ELISA) for the Nata analysis in foods.

Materials and methods

Reagents

Natamycin hydrochloride, tylosin tartrate, tilmicpsin, vancomycin hydrochloride, gentamycin sulfate, kanamycin sulfate, streptomycin sulfate, neomycin sulfate, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-Hydroxysuccinimide (NHS), and N, N' –Carbonyldiimidazole (CDI) were purchased from J&K Scientific Ltd. (Beijing, China). Bovine serum albumin (BSA), ovalbumin (OVA), Freund's complete and incomplete adjuvant, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES), 25% glutaraldehyde (GA) solution, 3,3',5,5'-tetramethylbenzidine (TMB), and polyethylene glycol 1500 (PEG 1500) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Enzyme immunoassay-grade horseradish peroxidase (HRP)-labelled goat anti-mouse immunoglobulin was supplied by Hua Mei Co. (Shanghai, China). RPMI-1640 cell culture medium, 50× HAT supplement

(containing hypoxanthine aminopterin thymidine), 100× HT supplement (containing hypoxanthine thymidine), and fetal bovine serum were obtained from Gibco BRL (Paisley, Scotland). All other chemicals and solvents were analytical grade.

Instruments

Absorbance measurements were fulfilled on a spectrophotometric microtiterplate reader (Thermo, MA, USA). An ultraviolet-visible spectrophotometer (Agilent, LA, USA) was used for UV spectra measurements. Centrifugations were implemented by a high-speed tabletop refrigerated centrifuge (Thermo, MA, USA). Milli-Q water purification system was purchased from Millipore (Bedford, MA).

Buffers and Solutions

The buffers and solutions used in this study have been described elsewhere [27]. The buffers and solutions included (1) coating buffer: 0.05 M carbonate buffer (CB, pH 9.6); (2) blocking buffer: 0.05 M CB with 0.2% gelatin (w/v); (3) washing buffer: 0.01 M PBS (pH 7.2) with 0.05% Tween-20 (v/v) (PBST); (4) assay buffer: 0.01 M HEPES (pH 7.2) with 5% methanol; (5) antibody diluent: 0.01 M PBS with 0.1% (w/v) gelatin and 0.05% (v/v) Tween 20; (6) substrate buffer: 100 mL of 0.1 M citrate phosphate buffer (pH 5.0) containing 18 μL of 30% H_2O_2; (7) TMB: 60 mg TMB dissolved in 100 mL ethylene glycol; (8) TMB substrate solution: 5:1 v/v mixture of substrate buffer and TMB solution; (9) 2 M H_2SO_4.

Synthesis of Immunogens and Coating Antigens

Due to the unique aliphatic amino group, the immunogens and coating antigens of Nata were synthesized through GA method, EDC method, and CDI method. Hence,

there were nine combinations of immunogens and coating antigens (shown in **Table 1**). For the GA method, 50 μL of 25% GA solution was added dropwise into 30 mg of Nata dissolved in 4 mL of methanol. The mixture solution was allowed to react for 20 min with stirring at room temperature prevented from light. Subsequently, the mixture was slowly added into 100 mg BSA/OVA dissolved in 5 mL PBS, allowed to react for 4 h at room temperature in dark, following with dialysis for 3 d using PBS. For EDC method, 30 mg of Nata dissolved in 4 mL of methanol was activated by adding with 13 mg of EDC and 12.6 mg of NHS for 1 h at room temperature kept from light. Then, the mixture was slowly added into 100 mg of BSA/OVA dissolved in 5 mL PBS to react for 8 h at room temperature in dark, following with dialysis for 3 d using PBS. For the CDI method, 30 mg of Nata dissolved in 4 mL of methanol without water was mixed with 6.8 mg of CDI to react for 30 min with at 37℃ prevented from light. The solution was added into 100 mg of BSA/OVA dissolved in PBS to form conjugates. The final solution was dialyzed for 3 d. The conjugates ratio was confirmed by UV-vis.

Immunization protocol

The immunization protocol was referred to standard schedule [28]. Briefly, eighteen BALB/c female mice were randomly divided into three groups; each group was subcutaneously injected at multiple points with one of three different immunogens (Nata-GA-BSA, Nata-EDC-BSA, and Nata-CDI-BSA). The first immunization was performed with 100 μg immunogen emulsified with Freund's complete adjuvant. Four weeks after the initial injection, booster immunizations were administered with a half mount of initial injection emulsified with Freund's incomplete adjuvant. After the third immunization, ic-ELISA was implemented to evaluate the titer and IC_{50} values

of mice sera. The mouse with the highest titer and lowest IC_{50} was screened as the spleen donor for cell fusion. About 20 d prior to cell fusion, a final intra-peritoneal injection (30 μg immunogens directly dissolved in 100 μL physiological saline) was implemented.

Cell Fusion and hybridoma screening

Cell fusion and hybridoma screening were performed according to standard protocol [29]. Briefly, spleen was rapidly removed from mouse and carefully ground to yield splenocytes, which were fused with mouse Sp2/0 myeloma cells under the effect of PEG 1500. The hybridoma cells were distributed into 96-well plates, cultivated by HAT medium containing 20% bovine fetal serum, and incubated for 7 d at 37°C and 5% CO_2. The supernatants were evaluated by ic-ELISA to screen the best cell line with the highest titer and the lowest IC_{50} values, which was used to generate sub-clones that were intraperitoneally administered to mice primed with paraffin. The ascites were purified by saturated-ammonium-sulfate method to obtain pure mAb. The concentrations of antibodies were evaluated by UV-vis spectroscopy at 278 nm. The antibodies were labeled and stored at -20°C for future use.

ic-ELISA

Bi-dimensional titration assays were performed to determine the most appropriate antibody concentration and the suitable coating antigen concentration for ic-ELISA [30]. Briefly, 100 μL of Nata-OVA (diluted with coating buffer to the concentrations of 1, 0.5, 0.25, 0.125, and 0.0625 μg L^{-1}) was coated on the microtiter plates at 37°C for 2 h. Then the microtiter plates were subsequently washed three times with PBST and added with 200 μL of blocking buffer at 37°C for 2 h. 50 μL of assay buffer and 50

μL of mAb (diluted with antibody diluent in 1:4000, 1:8000, 1:16000, 1:32000, 1:64000, and 1:128000) were added into each well and incubated for 30 min at 37°C. The antigen-antibody reaction was terminated by washing the microtiter plates three times with PBST, which followed by adding into each well with 100 μL of peroxidase-labelled goat anti-mouse IgG (diluted 3,000 times with antibody diluent) and incubating for 30 min at 37°C. The microtiter plates were washed for four times with PBST to remove the excess peroxidase-labelled goat anti-mouse IgG. 100 μL of TMB substrate solution was added into the plates. Finally, the enzymatic reaction was stopped with 50 μL of 2 M H_2SO_4 following with a 15-min incubation at 37°C. Optical density (OD) was measured at 450 nm on the microplate reader, and the results were plotted against the logarithm of analyte concentrations. As a result, the best combination of coating antigen concentration and antibody concentration were determined. The operation procedure of ic-ELISA was carried out according to standard schedule [31]. Briefly, the coating and blocking procedure were similar to the bi-dimensional titration assay. The difference was 50 μL of Nata (diluted with assay buffer to the concentrations of 0.3125, 0.625, 1.25, 2.5, 5, 10, and 20 μg L^{-1}) and 50 μL of mAb (diluted into the optimal concentration) were added into each well and incubated for 30 min at 37°C. The rest of steps were also similar to the bi-dimensional titration assay.

Sensitivity

IC_{50} was defined as the concentration of competing compound that produced a 50% inhibition of antibody binding to the coating antigen. Generally, it was regarded to be an indicator of mAb sensitivity. The limit of detection (LOD) was defined as the lowest concentration that exhibits a signal of 15% inhibition[32]. The detection range of

ic-ELISA was considered to be the concentration that resulted in 20–80% inhibition [30].

Specificity

The ability of structurally related analogues to combine with the mAb was defined as specificity. Generally, specificity was assessed by measuring cross-reactivity (CR), which was calculated according to the following equation [33],

$$CR\% = (IC_{50} \text{ value of Nata}) / (IC_{50} \text{ value of related compound}) \times 100.$$

The related analogues used to evaluate the specificity are included tylosin and tilmicosin, which are macrolide antibiotics; vancomycin, which is a glycopeptide antibiotics; gentamycin, kanamycin, streptomycin, and neomycin, which are aminoglycosides antibiotics. Among these analogues, tylosin, vancomycin, and four kinds of aminoglycosides antibiotics were dissolved in 0.01 M PBS for stock solution with concentration of 1 mg mL^{-1}. Tilmicosin was dissolved in DMF for stock solution. Each analogue was diluted with 0.01 M HEPES into different concentrations (5, 10, 20, 50, 100, 200, and 500 μg L^{-1}) for ic-ELISA.

Recovery test

Nata was fortified into negative milk, yoghurt, cheese, and juice for the recovery tests. For milk and juice samples, 3 mL of methanol was added into 1 mL of samples to allow the precipitation of protein under ultrasonic extraction for 30 min. The mixture was centrifuged at 875 rcf for 5 min; the resulting supernatant was filtered through a 0.22 μm microporous membrane to obtain the pure liquid, which was diluted ten times to the final concentrations (1, 2, and 5 μg L^{-1}) for the ic-ELISA. As semisolid samples, negative yoghurt and cheese were firstly homogenized and then spiked with

Nata, following with the similar ultrasonic extraction process like milk and juice. After extraction, 1 mL of pure water was added into the mixture and kept in 4°C for 1h, following with the centrifugation. After a microfiltration, the extraction was diluted to the final concentrations (1, 2, and 5 μg L^{-1}) for the ic-ELISA.

Results and discussion

Antigen conjugation

To be immunogenic, small molecules such as Nata have to be covalently coupled with a carrier protein, including BSA, KLH, and OVA [34]. BSA and KLH are usually used for the conjugation of immunogens; OVA is often used for the conjugation of coating antigens. Nata is a polyene macrolide, which contains four alkenes in an annular structure. Nata is completely stable under the pH value of most food products; and the solubility of Nata will increase under pH > 9 or pH < 3. In addition, it is relatively stable under dry conditions and even can tolerate high temperatures for short time. However, due to its annular structure, Nata is relatively sensitive to ultraviolet rays. Hence, it is crucial to prevent Nata from light. To confirm successful conjugation, UV absorbances recorded from 200 to 500 nm of Nata, BSA, OVA, and their conjugates were measured, respectively. The maximum absorbance peak of BSA is at 278 nm generated from its aromatic group and Nata has three obvious characteristic absorbance peaks at 292, 305, and 320 nm, which generated by the structure of annular four alkenes. Deservedly, the conjugates should possess the characteristic absorbance peaks of Nata cause that the superfluous Nata has been removed through dialysis. As shown in **Figure 1**, conjugates were successfully synthesized by the three different methods. Generally speaking, the GA method is frequently applied for the conjugation between aliphatic amino group and carrier protein and it is the more

effective compared with EDC and CDI method. The ratio of conjugation through three different methods were 14, 2, and 2.2, respectively.

As shown in **Table 1**, each kind of antiserum was evaluated with three different coating antigens (Nata-GA-OVA, Nata-EDC-OVA, and Nata-CDI-OVA). Apparently, the titer of antiserum (GA) is the highest, which caused by the high coupling ratio between Nata and BSA. Glutaraldehyde, a common homobifunctional crossing-linking agent, has been widely used in chemical synthesis [35]. The amino group of Nata was aliphatic, so it is proper to active Nata using glutaraldehyde and result in the high conjugate ratio. Simultaneously, the inhibition of antiserum (GA) was optimum when Nata-GA-OVA or Nata-EDC-OVA was used as the coating antigen.

Optimization of ic-ELISA

The ionic strength, pH values, and organic solvent content of the assay buffer could affect protein conformation and significantly influence the binding of antigen to antibody [36]. As shown in **Figure 2 (A)** and **(B)**, comparing with using 0.01 M PBS, ic-ELISA had a higher titer and lower IC_{50} values using 0.01 M HEPES to dilute Nata. Organic solvent was often used to assist the hydrophobic analytes to dissolve in sample buffer, while high concentrations of organic solvent can negatively affect the properties of mAb (e.g., mAb sensitivity) owning to the presence of background and nonspecific binding. As shown in **Figure 2 (B)**, the concentration of methanol had little effect on the maximum optical density (OD). With 20% methanol (v/v) in 0.01M HEPES, the sensitivity was poor due to antibody deactivation. With 5% methanol (v/v) in 0.01M HEPES used as assay buffer, the ic-ELISA had the highest OD and the lowest IC_{50} values. As shown in **Figure 2 (C)**, the OD was less than 1 when pH value

was 9.6, while the OD was higher and IC_{50} was lower than others under pH 7.2, which indicated that pH 7.2 was the most optimal one.

The standard sigmoidal inhibition curve of Nata under the optimized conditions was shown in **Figure 2 (D)**. The IC_{50} value and the quantitative detection range (IC_{20}–IC_{80}) was 1.69 and 0.64–4.46 μg L^{-1}**,** respectively. The limit of detection (LOD) of the method was 0.59 μg L^{-1}.

Cross-reactivity

As shown in **Table 2**, the seven analogues almost have no cross-reactivities with Nata, which indicated that the mAb against Nata was specific.

Recovery tests

For liquid samples (e.g., milk and juice) were diluted 4 times during the extraction process, while the semisolid samples (e.g., yoghurt and cheese) were diluted 5 times. The supernatant was diluted ten times to eliminate the matrix interferences. As shown in **Table 3**, the average recoveries of Nata fortified in negative milk, juice, yoghurt, and cheese was 103–121%, 103–121%, 84–114%, and 89–108%, respectively. The results revealed that the mAb against Nata can be applied for the detection of Nata in milk, juice, yoghurt, and cheese using ic-ELISA.

Conclusions

In this study, a highly sensitive and specific mAb against Nata was developed for the first time and it can satisfy the Nata detection of European Union. Subsequently, a simple and rapid ic-ELISA for Nata detection in milk, juice, yoghurt, and cheese, was established, which can be employed for commercial ELISA kits and colloidal gold

immunochromatographic strips.

Acknowledgements

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Captions:

Fig. 1 The UV-Vis absorption spectra of Nata-BSA (A) and Nata-OVA (B). The concentrations of Nata-BSA (Nata-GA-BSA, Nata-EDC-BSA, and Nata-CDI-BSA) and Nata-OVA (Nata-GA-OVA, Nata-EDC-OVA, and Nata-CDI-OVA) were both 0.5 mg mL^{-1}.

Fig. 2 Optimization of assay buffer for ic-ELISA: (A) Effect of methanol content in PBS on ic-ELISA performance; (B) Effect of methanol content in HEPES on ic-ELISA performance; (C) Effect of pH value of assay buffer on ic-ELISA performance; (D) Standard curve of inhibition. Coating antigen: Nata-GA-OVA, 0.0625 μg mL^{-1}; antibody: 1:64000; standards: 0.3125, 0.625, 1.25, 2.5, 5, 10, and 20 μg L^{-1}. Each point represents the mean of ±SD of three replicates.

Table 1 Titer and inhibition of nine combinations of immunogens and coating antigens.

Table 2 Cross-reactivity of the mAb

Table 3 Recovery test for Nata spiked in milk, juice, yoghurt, and cheese.

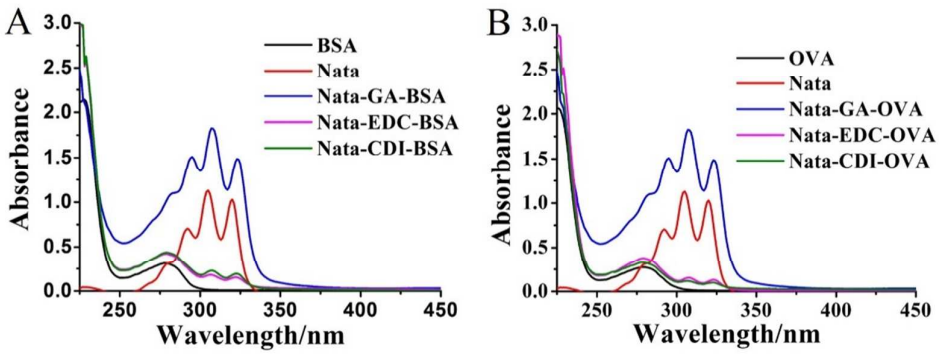

Fig. 1 The UV-Vis absorption spectra of Nata-BSA (A) and Nata-OVA (B). The concentrations of Nata-BSA (Nata-GA-BSA, Nata-EDC-BSA, and Nata-CDI-BSA) and Nata-OVA (Nata-GA-OVA, Nata-EDC-OVA, and Nata-CDI-OVA) were both 0.5 mg mL^{-1}.

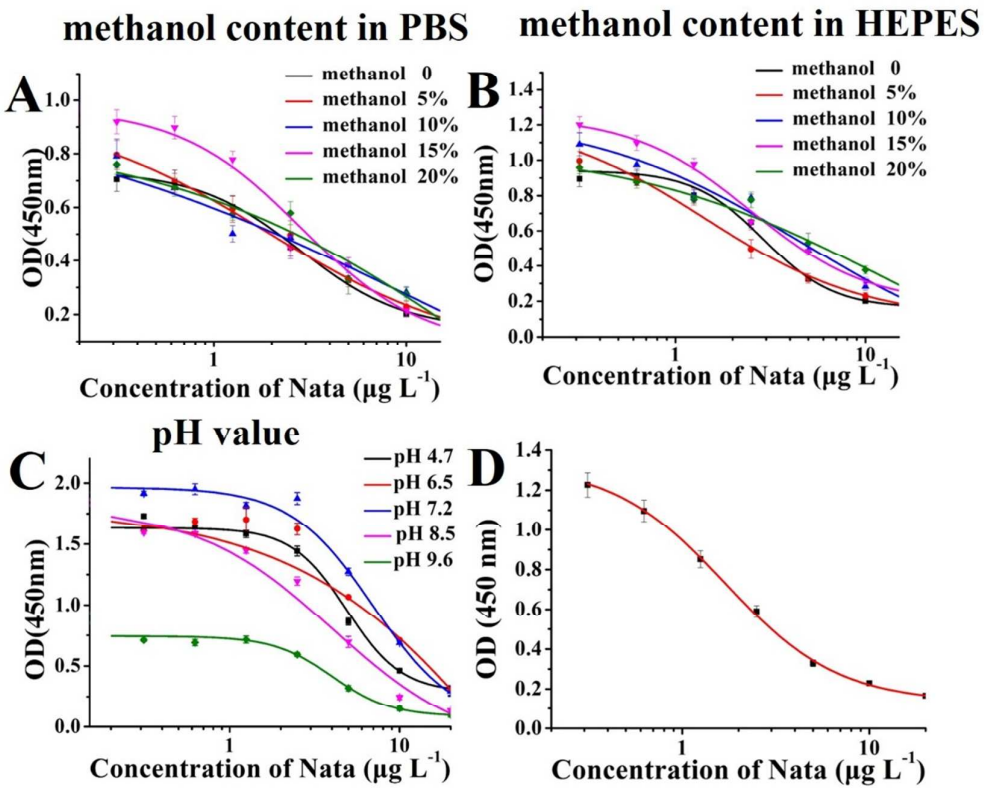

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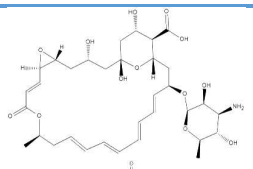
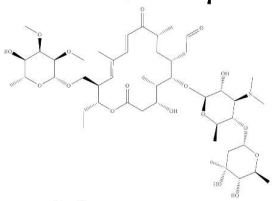
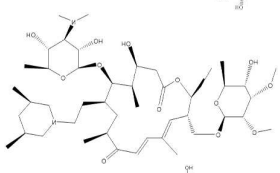
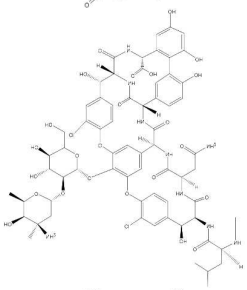
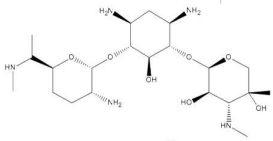
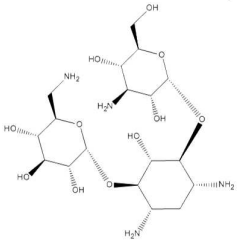
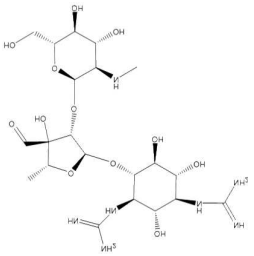
Table 1 Titer and inhibition of nine combinations of immunogens and coating antigens

coating antigen	antiserum(GA) titer[a]	antiserum(GA) inhibition[b]	antiserum(EDC) titer	antiserum(EDC) inhibition	antiserum(CDI) titer	antiserum(CDI) inhibition
	($\times10^3$)	(%)	($\times10^3$)	(%)	($\times10^3$)	(%)
Nata-GA-OVA	9	45	6	41	<1	39
Nata-EDC-OVA	9	60	8	52	<1	23
Nata-CDI-OVA	<1	10	<1	15	6	10

[a]Titer is defined as dilution factor of antiserum in the absorbance at 450 nm under the coating concentration of 0.1 μg L^{-1}.

[b]Inhibition ration was calculated as follow: inhibition (%) = [1-(B / B_0)] ×100. B_0 was mean value of absorbance of wells without competitor; B was mean value of absorbance of wells in the presence of competitor (10 μg L^{-1}).

Table 2 Cross-reactivity of the mAb

Compound	Structure	IC_{50} ($\mu g\ L^{-1}$)	Cross-reactivity (%)
Natamycin		1.69	100
Tylosin		>500	<0.1
Tilmicosin		>500	<0.1
Vancomycin		>500	<0.1
Gentamycin		>500	<0.1
Kanamycin		>500	<0.1
Streptomycin		>500	<0.1

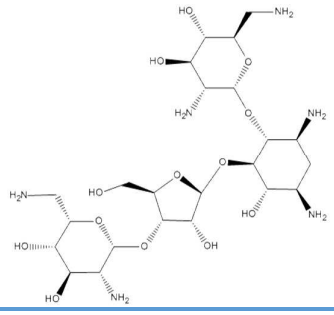
Neomycin		>500	<0.1

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Table 3 Recovery test for Nata spiked in milk, juice, yoghurt, and cheese.

Matrix	Spike Nata ($\mu g\ L^{-1}$)	Intraassay Mean±SD ($\mu g\ L^{-1}$)	Intraassay Recovery (%)	Intraassay CV (%)	Interassay Mean±SD ($\mu g\ L^{-1}$)	Interassay Recovery (%)	Interassay CV (%)
Milk	1	1.20 ± 0.05	120.0 ± 5.0	4.17	0.94 ± 0.03	94.0 ± 3.0	3.19
	2	2.19 ± 0.15	109.5 ± 7.5	6.85	2.05 ± 0.03	102.5 ± 1.5	1.46
	5	5.13 ± 0.40	102.6 ± 8.0	7.80	4.92 ± 0.03	98.4 ± 0.6	0.61
Juice	1	1.21 ± 0.02	121.0 ± 2.0	1.65	1.09 ± 0.05	109.0 ± 5.0	4.59
	2	2.37 ± 0.03	118.5 ± 1.5	1.27	2.13 ± 0.03	106.5 ± 1.5	1.41
	5	5.05 ± 0.05	101.0 ± 1.0	0.99	4.95 ± 0.03	99.0 ± 0.6	0.61
Yoghurt	1	0.93 ± 0.04	93.0 ± 4.0	4.30	0.89 ± 0.03	89.0 ± 3.0	3.37
	2	2.27 ± 0.03	113.5 ± 1.5	1.32	1.85 ± 0.04	92.5 ± 2.0	2.16
	5	4.19 ± 0.02	83.8 ± 0.4	0.48	4.51 ± 0.04	90.2 ± 0.8	0.89
Cheese	1	0.90 ± 0.03	90.0 ± 3.0	5.17	0.88 ± 0.03	88.0 ± 3.0	3.82
	2	2.16 ± 0.04	108.0 ± 2.0	3.26	1.83 ± 0.03	91.5 ± 1.5	4.78
	5	4.43 ± 0.02	88.6 ± 0.4	4.62	4.64 ± 0.04	92.8 ± 0.8	2.96