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Neamin as an Immunogen for the Development of a Generic ELISA Detecting Gentamicin, Kanamycin, and Neomycin in Milk

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A broad-specific ELISA using one antibody preparation for the detection of gentamicin, kanamycin, and neomycin in milk is reported for the first time. For the immunization of rabbits, neamin was used as the generic hapten on the basis of the facts that it is a two-ring fragment of neomycin and, in shape and charge, it resembles parts of kanamycin and gentamicin. Neamin was linked to the preactivated carrier protein keyhole limpet hemocyanin by EDC coupling. The specificity and sensitivity of the polyclonal antibodies for the aminoglycoside antibiotics were tested in a competitive assay using homologous and heterologous conjugates coupled by various conjugation procedures as the ELISA solid phase. In contrast to the homologous assay recognizing only neomycin, the heterologous assay could be used for the detection of the whole subclass of deoxystreptamin antibiotics in buffer and raw milk. Gentamicin, kanamycin, and neomcyin were detected in artificially contaminated undiluted raw milk (without sample pretreatment) with 50% inhibition levels at 9, 21, and 113 ng mL^{-1}, respectively. Neomycin levels were also measured in milk samples obtained from a cow suffering from mastitis and treated with an antibiotic cocktail including neomycin. Levels below the EU maximum residue levels for deoxystreptamin antibiotics could readily be measured in this generic ELISA.

KEYWORDS: Antibiotic; aminoglycoside; gentamicin; kanamycin; neomycin; dihydrostreptomycin; neamin; generic hapten; generic antibody; ELISA; milk

INTRODUCTION

The health risks that urge the detection of antibiotics in food and food products have a pharmacological, toxicological, microbiological (in relation to resistant or pathogenic microorganisms in the intestinal flora), or immunopathological (e.g., allergies) nature (*3*). In this respect, the contamination of milk and milk products with antibiotics is being monitored in most European countries. The control system is related to two different aspects, that is, payment for milk on the basis of quality and public health as governed by food and drug legislation. These aspects are of variable importance in different countries. The primary aim is to monitor the antibiotic residues in the context of examination for compliance with EU legislation on maximum residue levels (MRLs) in milk and milk products as detailed in Council Regulations (EEC). Aminoglycosides are one of the major classes of antibiotics used in veterinary treatment. For the class of aminoglycosides, the following MRLs have been set for milk: 100 μg kg^{-1} (ng mL^{-1}) for gentamicin, 150 μg kg^{-1} for kanamycin, 200 μg kg^{-1} for (dihydro)-streptomycin, and 500 μg kg^{-1} for neomycin (*1*). Gentamicin, kanamycin, and neomycin belong to the subclass (i.e., group) of deoxystreptamin antibiotics. (Dihydro)streptomycin [(DH)-streptomycin] belongs to the group of streptidine antibiotics.

Microbial inhibition tests (e.g., the Brilliant black reduction test, Delvotest) are frequently employed in the first line of screening for antimicrobial drug residues in milk. These types of tests detect a very broad spectrum of antibiotics in an inexpensive and simple way. However, the aminoglycosides often cannot be detected at their MRL levels. When a milk sample turns out to be positive in the first line of screening and cannot be assigned to either the sulfonamides or β-lactams in additional conformational group tests, The Netherlands Milk Control Station (Zutphen, The Netherlands) employs a radioreceptor assay (i.e., Charm II Aminoglycoside test; Charm Sciences, Malden, MA) to assess the presence of deoxystreptamine antibiotics. A recently introduced multiplate microbial inhibition assay shows increased sensitivity for the aminoglycosides in milk (25 μg kg^{-1} (ng mL^{-1}) for gentamicin, 150 μg kg^{-1} for kanamycin, 100 μg kg^{-1} for (dihydro)-streptomycin, and 50 μg kg^{-1} for neomycin) (*2*).

Physicochemical methods (e.g., thin-layer chromatography and high-pressure liquid chromatography), on the other hand, are able to detect all aminoglycosides at MRL level but lack group-specific detection and the possibility for routine screening.

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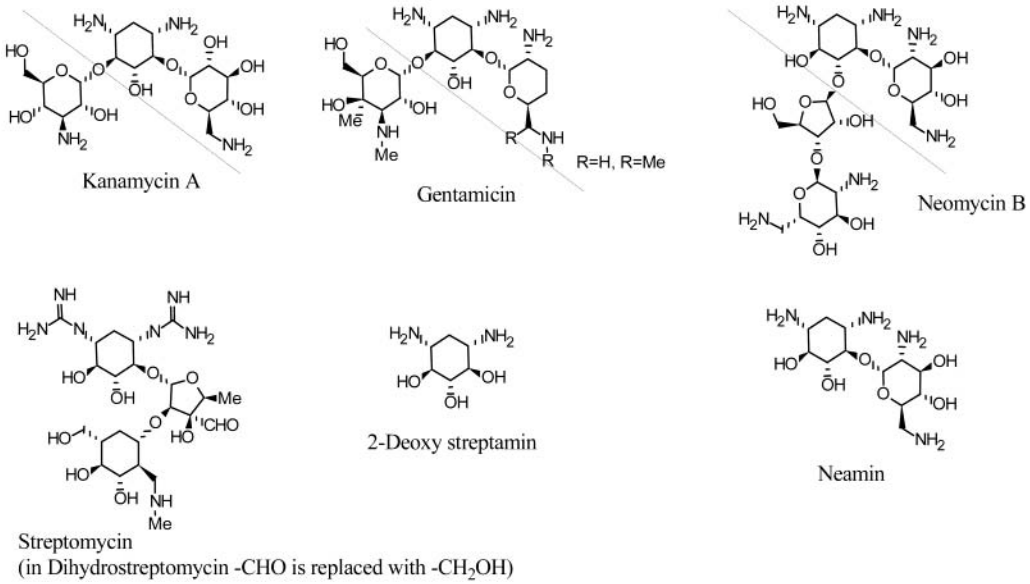

Figure 1. Structures of the aminoglycosides kanamycin A, gentamicin, neomycin B, and streptomycin. In addition, the two-ring structure of neomycin, the so-called neamin, and the deoxystreptamin nucleus common to the deoxystreptamin antibiotics are shown.

Potentially, immunochemical assays could circumvent all of these drawbacks if suitable antibodies were available.

In the past decade, immunochemical methods have become increasingly important in antimicrobial drug monitoring (*3*, *4*). Potentially, both on- and off-farm markets can be covered by immunochemical test methods. Notably, immunochemical tests to detect β-lactams have found extensive commercial exploitation. In the category of the aminoglycosides, only a small number of competitive enzyme immunoassays (EIAs) are commercially available that specifically detect kanamycins, gentamicins, neomycins, or DH-streptomycin in milk (e.g., gentamicin EIA, neomycin EIA, and streptomycin EIA from Eurodiagnostica, Malmö, Sweden; Ridascreen Streptomycin ELISA from R-Biopharm, Darmstadt, Germany). In the literature, several immunochemical assays that specifically detect kanamycins, gentamicins, neomycins, or DH-streptomycin in milk have been reported (*5*−*10*). However, no immunoassay has been reported so far using one antibody preparation to recognize structurally different aminoglycosides at the same time.

The aim of the present work was to develop antibodies that are able to recognize, as much as possible, the whole class of aminoglycoside antibiotics. To raise class-specific, so-called generic antibodies, several approaches can be followed. A first possibility is to combine residue-specific poly- or monoclonal antibodies that together ensure the recognition of a whole class of antibiotic residues (*11*, *12*). A second possibility is the combination of specific recombinant antibody fragments. Recently, manipulation of the antibody binding site (and hence manipulation of antibody specificity and/or affinity) has become feasible. This might lead to adaptation of the recognition spectrum to class-specific antibody-fragments.

Alternatively, a generic residue having a chemical structure common to all or most members of a particular class of antibiotic residues can be used as the immunogen. Usleber and colleagues have been successful in raising group-specific antibodies recognizing penicillins by the use of ampicillin as a specific β-lactam residue (*13*, *14*). For the class of sulfonamides, this approach has brought variable success (*16*−*18*). We have not found such an approach for the aminoglycoside antibiotics in the literature.

Unfortunately, the aminoglycosides represent a class of antibiotics of which the individual components show only limited structural similarity. All aminoglycosides contain multiple amino groups, but the sizes, structures, and charges of the individual compounds vary to a large extent. Not surprisingly, antibodies raised to immunogens containing specific aminoglycoside residues have been reported to be fairly specific (*8*).

The four aminoglycosides that were selected to represent this class of compounds were kanamycin, neomycin, gentamicin, and DH-streptomycin (**Figure 1**). These four members are the most important aminoglycoside antibiotics used as therapeutics in veterinary medicine in the European Union. Three of these members represent the subclass of deoxystreptamin antibiotics, and all contain the central 2-deoxy streptamine ring; only DH-streptomycin does not. DH-streptomycin belongs to the subclass of streptidine antibiotics. In both kanamycin and gentamicin, the central ring is 1,3-substituted, whereas in neomycin, it is 1,2-substituted. Molecular modeling simulations showed that kanamycin and gentamicin are quite similar in structure and shape, with the (charged) amino groups occupying similar positions. Neomycin also fitted in these structures with its upper two rings. On the basis of these observations, we decided to use a compound consisting of the upper two rings of neomycin, i.e., neamin, as the generic hapten. This two-ring fragment contains the central 2-deoxy streptamine ring present in kanamycin and gentamicin as well and, therefore, could serve as a generic hapten for the subclass of deoxystreptamine antibiotics within the class of aminoglycoside antibiotics.

MATERIALS AND METHODS

Materials. Tween 20, *p*-nitrofenyl phosphate, and glutaraldehyde were obtained from Merck (Darmstadt, Germany). Bovine serum albumin (BSA) and neamin were acquired from ICN Biomedicals (Aurora, OH). Gentamicin sulfate and kanamycin sulfate were purchased from Duchefa (Haarlem, The Netherlands). Neomycin trisulfatehydrate and dihydrostreptomycin sesquisulfate were obtained from Fluka (Buchs, Switzerland). Flat-bottom microtiter ELISA plates (EIA/RIA high-binding plates, 96 wells) were from Costar (Schiphol-Rijk, The Netherlands). *N*-Ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC) hydrochloride, goat anti-rabbit immunoglobulin, Freund's complete and incomplete adjuvants, alkaline phosphatase conjugated to goat anti-rabbit immunoglobulin, and keyhole lympet hemocyanin

(KLH) were obtained from Sigma-Aldrich (Steinheim, Germany). All other chemicals were of analytical-grade quality. The Visking dialysis tubing (12.000−14.0000 D) was supplied by Medicall International Ltd. (London, United Kingdom) and the protein G column (Sepharose 4 Fast Flow LKB-GP-10) by Amersham Pharmacia Biotech AB (Roosendaal, The Netherlands). An ultrafiltration cell (model 8050) was supplied by Amicon Grace Company (Beverly, MA) and the BCA kit by Pierce (Rockford, IL). *N*-Hydroxysuccinimide (NHS) was also from Pierce (Rockford, IL).

Equipment. The microtiter plates were washed with a microtiter plate washer (Anthos Labtec Instruments GmbH, Salzburg, Austria). The absorbance was read with a microtiter plate reader (Microplate Reader model 3550-UV from Biorad Laboratories, Veenendaal, The Netherlands).

Synthesis of Immunogen. The immunogen was prepared by coupling neamin to preactivated KLH through the activated carbodiimide coupling method (subsequently referred to as the ACC method).

Preactivation of the carrier protein was performed with succinic anhydride to introduce additional coupling sites and to reduce the chance of creating interprotein cross-links. Subsequently, the conjugation reaction between the protein carboxyl groups and the amino groups of neamin was performed using the water-soluble carbodiimide reagent EDC.

To produce *N*-hydroxysuccinimide succinate, necessary for the preactivation of the carrier protein, 5 g of NHS (43 mmol) and 4.35 g of succinic anhydride (1 equiv) were dissolved in 30 mL of dichloromethane, and 7.2 mL of triethylamine (1.2 equiv) was added. The reaction mixture was stirred for 3 h at room temperature and mixed with 50 mL of ethyl acetate in a separatory funnel. The organic phase was washed twice with 25 mL of ice-cold 1 N HCl, dried over sodium sulfate, and concentrated under vacuum. An off-white solid (approximately 1 g) was obtained that was used without further purification.

Of a stock solution of KLH (10 mg mL^{-1}) in phosphate-buffered saline (PBS; 15 mM, 0.8% NaCl, pH 7.4), 200 μL was transferred to an Eppendorf vial and mixed with 800 μL of PBS. *N*-Hydroxysuccinimide succinate was dissolved in DMF (10 mg mL^{-1}), and 60 μL was added to the KLH solution. The reaction mixture was vortexed and incubated at room temperature for 45 min. Another 60 μL of the same *N*-hydroxysuccinimide succinate solution was added, and the reaction mixture was vortexed and incubated for another 45 min at room temperature. The solution was transferred to a Biomax concentrator (Millipore, Bedford, MA, cutoff 30,000) and concentrated by centrifugation (30 min, 3000 g, 4 °C). One milliliter of a 5 mM MES buffer (pH 5) was added, and the solution was centrifuged once more. This step was repeated twice to ensure complete buffer exchange. The activated protein solution was taken up in 1 mL of MES buffer for the conjugation reaction with neamin.

Neamin was dissolved in the MES buffer (10 mg mL^{-1}), and 200 μL of this solution was added to the activated protein solution. EDC was dissolved in the same buffer (10 mg mL^{-1}; freshly made), and 200 μL of the resulting solution was added to the solution containing both protein and neamin. The reaction mixture was vortexed, incubated at room temperature for 2 h, transferred to a Biomax concentrator, and concentrated by centrifugation (30 min, 3000 g, 4 °C). One milliliter of PBS (pH 7) was added, and the solution was centrifuged once more. This step was repeated twice to ensure complete buffer exchange. The protein solution was diluted with PBS to give a 1 mg mL^{-1} solution that was used as such for immunization.

Synthesis of ELISA Solid-Phase Antigen Conjugates. A BSA−neamin conjugate coupled by the ACC method was prepared as indicated in the section Synthesis of Immunogen. As alternative ELISA solid-phase (i.e., coating) conjugates, other aminoglycosides (gentamicin, kanamycin, neomycin, or DH-streptomycin) were coupled to BSA as well. For this linkage, the glutaraldehyde (GDA) coupling method was performed at two pH values (7.3 and 8.5) because of observed differences in solubility at these pH values. BSA (2 mg mL^{-1}) was dissolved in 0.1 M sodium carbonate (containing 0.15 M NaCl, pH 8.5), and subsequently, one of the aminoglycosides was added to the protein solution to obtain a concentration of about 2 mg mL^{-1}. After freshly prepared glutaraldehyde [1% (v/v) final concentration] had been added, the reaction mixture was vortexed and incubated for 3 h at 4 °C while using a gentle rocker. Sodium borohydride was then added to a final concentration of 10 mg mL^{-1}, and the solution was incubated for 1 h at 4 °C. Finally, the conjugate was purified by gel filtration or dialysis to remove excess reagents.

Immunization of Rabbits. Two New Zealand White rabbits (SN329 and SN330) were subcutaneously immunized with 100 μg of conjugate (KLH−neamin) in 1 mL PBS/cFA (1:1). The first two boosters were subcutaneously injected at 2-week intervals with 50 μg of conjugate in 1 mL of PBS/iFA (1:1). All other boosters were given at 4-week intervals. One week after each booster, serum titers were determined in ELISA. The antiserum obtained after each booster was prepared by allowing the blood to clot overnight at 4 °C, followed by centrifugation to remove particulate material. A portion of the final serum of rabbit SN329 was purified by protein G chromatography equipment in accordance with the manufacturer's instruction manual.

ELISA. The microtiter plates were freshly coated for each experiment. To prevent any evaporation, the wells were sealed during every incubation and washed three times afterward with PBS/Tween [0.05% (v/v) Tween 20, pH 7.4]. ELISAs were performed as follows:

Polystyrene microtiter plate wells (Costar) were coated, during an overnight incubation at 4 °C or a 1-h incubation at 37 °C, with 100 μL of an aminoglycoside−BSA conjugate at different coating concentrations (maximum concentration of 1 μg $well^{-1}$) in 0.05 mol L^{-1} carbonate/bicarbonate coating buffer (pH 9.6). Plates were emptied, and nonspecific adsorption of the antibody was prevented by performing an incubation for 2 h at 37 °C with 200-μL aliquots of bovine serum albumin or casein [2% (w/v) in PBS, pH 7.4]. After being washed, the serum was diluted in dilution buffer [PBS plus 0.1% (w/v) bovine serum albumin or casein] of which 100 μL was transferred to the wells of the microtiter plate, and the plate was incubated for 1 h at 37 °C. After being washed, 100 μL of the secondary antibody conjugate (goat anti-rabbit IgG labeled with alkaline phosphatase), in a dilution of 1:1000 in dilution buffer, was added, and the plate was incubated for 1 h at 37 °C. The final washing procedure was followed by a color development, which was initiated by adding 200 μL of the substrate solution (4-nitrophenyl phosphate disodium salt, 1 mg mL^{-1} in 0.05 mol L^{-1} carbonate/bicarbonate buffer plus 100 ng L^{-1} $MgCl_2 \cdot H_2O$, pH 9.6). The optical density of each well was recorded at 405 nM with a plate reader after various time intervals.

For the determination of the relative antibody titer of the rabbit sera, a coating conjugate concentration of 1 μg mL^{-1} was used. The titer was defined as the antiserum dilution that resulted in 1.0 absorbance units (*13*). The preimmune control sera gave maximum absorbance values of <0.2 units under these conditions.

For the determination of the specificity and sensitivity of the anti-neamin serum, the ELISA described above was used in a competitive format with free aminoglycosides. After the coated and blocked plate had been washed, 50 μL of the aminoglycoside solution was added to the well directly, followed by the diluted antiserum. The plates were further treated as described before.

The intra-assay coefficients of variation were usually below 8%, whereas the inter-assay coefficients of variation were usually below 12%. Data points in graphics are expressed as differences between the optical density of the standard/sample and the optical density of the blank signal. Curve fitting and IC_{50} value determination (concentration of inhibitor that leads to a 50% decrease of the maximum signal) were performed using the Life Science Workbench (LSW) Data Analysis Toolbox from MDL Information Systems, Inc., in Microsoft Excel.

Determination of Aminoglycosides in Milk Samples. Raw milk was collected from a tank on a farm in the province Zeeland (The Netherlands). The raw milk was stored for a maximum of 3 days at 4 °C before use in ELISA. This raw milk from untreated cows was artificially contaminated with aminoglycosides in powder form (gentamicin, kanamycin, neomycin, and DH-streptomycin) directly dissolved in milk.

Six milk samples from treated cow 040036 were obtained from The Netherlands Controlling Authority for Milk and Milk Products. This cow, from the experimental herd of the Research Centre for cattle, sheep, and horses (The Netherlands), suffered from mastitis and was treated by (intramammary and parental) injections with a preparation containing amoxicillin, penicillin, and neomycin on three subsequent

days (June 25−28, 1998). Collection of six milk samples took place by milkings at two different time intervals in the three following days after treatment (June 29−July 1, 1998). Shortly after collection and storage at 4 °C, the amount of neomycin in these six milk samples was determined by the Charm II Aminoglycosides test. Subsequently, these samples were stored at −20 °C. Samplings containing a neomycin concentration above the MRL or samples collected before the start of the treatment could not be obtained. In competition ELISA, all samples (1:1 diluted in 2× concentrated buffer) were tested in duplicate, including blanks, although sample dilution or any pretreatment of the raw milk samples was not necessary before use in ELISA.

A negative milk sample (confirmed by the Charm II Aminoglycosides test) from an untreated cow (120648) served as the noncompetitor control sample (i.e., provided the maximum OD signal in the competition ELISA).

When undiluted raw milk was applied in the competition ELISA, the antiserum was diluted in milk as well.

RESULTS

The aim of this study was to produce a polyclonal antiserum that recognized most members of the class of aminoglycoside antibiotics. Gentamicin, kanamycin, neomycin, and DH-streptomycin were selected as representative members. Neamin, a two-ring fragment of neomycin, was chosen to prepare the immunogen (**Figure 1**).

The two rabbits immunized with KLH−neamin both developed good antibody titers, as was measured in ELISA using BSA−neamin as the coating conjugate. The antibodies present in the bleedings obtained after the third booster (day 66) also bound to all heterologous coating conjugates, i.e., several aminoglycoside−BSA conjugates. For both rabbits, the highest titer (1:8000) was found when using the BSA−neamin conjugate in ELISA. The polyclonal antibodies produced by rabbit SN329 showed higher cross-reactivities toward the aminoglycosides and better sensitivity than the antibodies of rabbit SN330. Therefore, only competition results with serum from rabbit SN329 are described. Displacement of bound anti-neamin antibodies (the serum obtained after the third booster, day 66) could be observed only when using the following four different coating conjugates in ELISA: BSA−neamin ACC (16 ng well^{-1}), BSA−neomycin ACC (32 ng well^{-1}), BSA−neomycin GDA 7.3 (500 ng well^{-1}), and BSA−kanamycin GDA 8.5 (250 ng well^{-1}). **Figure 2** (upper panel) shows that, with the BSA−neamin conjugate on the plate, significant displacement was observed only with free neamin (IC50 of 8 ng mL^{-1}) and neomycin (IC50 of 1900 ng mL^{-1}). A better sensitivity toward more aminoglycosides could be observed using the other coating conjugates such as BSA−kanamycin GDA 8.5 and BSA−neomycin GDA 7.3 (heterologous approach). The latter gave the most sensitive displacement results, and the 50% inhibition values in buffer were 0.07, 0.14, 8, 13, and 3940 ng mL^{-1} for free neamin, neomycin, gentamicin, kanamycin, and DH-streptomycin, respectively (**Figure 2**, lower panel).

A portion of the final serum, taken after 9 boosters at day 332, of rabbit SN329 was purified by protein G chromatography.

An extensive study, using these protein G purified polyclonal antibodies and different coating conjugates on the plate, showed binding of the antibodies to all available BSA−aminoglycoside conjugates, except for BSA−neomycin (ACC) (**Figure 3**).

In contrast to the results obtained with the unpurified serum of day 66, the binding of the affinity-purified polyclonal antibodies, prepared from the serum of day 332, could no longer be displaced by competition with neamin and neomycin using the homologous coating conjugate (BSA−neamin) in the ELISA. Of all heterologous coating conjugates, BSA−kanamycin (GDA 8.5) and BSA−neomycin (GDA 7.3) resulted in the most sensitive displacement. The best results were obtained with the BSA−kanamycin (GDA 8.5) coating, which resulted in 50% inhibition values of 15, 19, and 152 ng mL^{-1} for gentamicin, kanamycin, and neomycin, respectively (**Figure 4**, upper panel). With DH-streptomycin no significant displacement was observed.

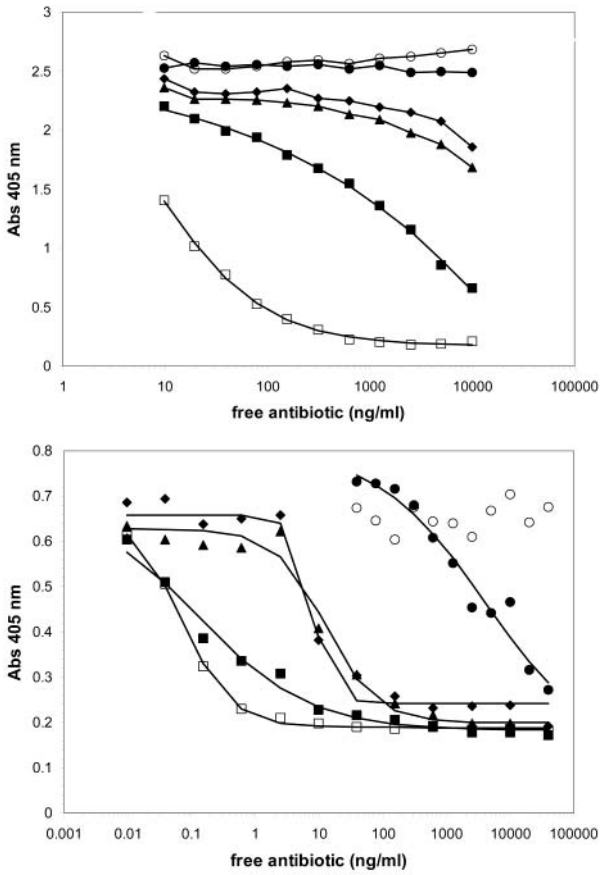

Figure 2. ELISA calibration curves obtained with the unpurified anti-neamin serum of day 66 from a KLH–neamin ACC-immunized rabbit SN329 on a BSA–neamin ACC-coated plate (upper panel) and a BSA–neomycin (GDA 7.3)-coated plate (lower panel) with the following compounds as competitors: neomycin, ■; DH-streptomycin, ●; kanamycin, ▲; gentamicin, ◆; neamin, □; 6-aminopenicillanic acid, ○. Data represent means from duplicate wells.

The applicability of this generic ELISA for the determination of neomycin, kanamycin, and gentamicin in milk was examined by analyzing artificially contaminated milk. From initial experiments (data not shown), it could be concluded that sample pretreatment of raw milk and other milk types (full-fat, frozen), such as centrifugation or dilution, was not necessary for the determination of these aminoglycosides at their MRL levels. **Figure 4** (lower panel) shows the displacement results of the anti-neamin antiserum in undiluted raw milk by the three aminoglycosides, and the 50% inhibition values were 9, 21, and 113 ng mL^{-1} for gentamicin, kanamycin, and neomycin, respectively. The optical density values as well as the 50% inhibition values were almost comparable to the results obtained in buffer (**Figure 4**, upper panel). **Table 1** shows the characteristics of the purified anti-neamin serum in buffer and undiluted raw milk.

Six milk samples were obtained by sampling one sick cow at two different time intervals on three subsequent days following injections with preparations containing penicillin, amoxicillin, and neomycin. **Figure 4** (lower panel) shows the

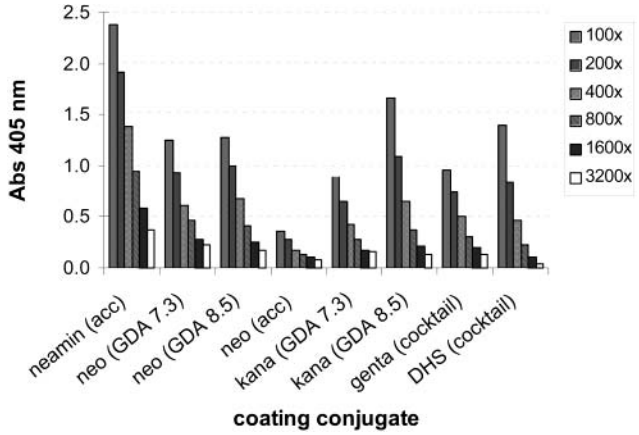

Figure 3. ELISA reactivity of the protein G purified final anti-neamin serum (SN329) in various dilutions on various BSA–hapten coating conjugates (homologous and heterologous) using ELISA. BSA–neamin coupled by the activated carbodiimide method (neamin ACC); BSA–neomycin coupled by the glutaraldehyde method at pH 7.3 (neo GDA 7.3); BSA–neomycin coupled by the glutaraldehyde method at pH 8.5 (neo GDA 8.5); BSA–neomycin ACC (neo ACC); BSA–kanamycin GDA 7.3 (kana GDA 7.3); BSA–kanamycin GDA 8.5 (kana GDA 8.5); BSA–gentamicin coupled by ACC, GDA 7.3, and GDA 8.5 (gentamicin cocktail), BSA–dihydrostreptomycin coupled by ACC, GDA 7.3, and GDA 8.5 (DH-streptomycin cocktail).

neomycin calibration curve, using spiked and undiluted raw milk samples, that was used to quantify the amount of neomycin in the acquired milk samples. When the neomycin concentration in the milk samples was 300 ng mL^{-1} (according to the Charm II Aminoglycosides test), the results of the ELISA and the Charm test were comparable (**Table 2**). When the concentration of neomycin in the milk samples was 100 ng mL^{-1} or lower (according to the Charm II Aminoglycosides test), the ELISA results obtained were 34−95% higher in comparison to the Charm test.

DISCUSSION

For the development of antibodies recognizing most members of a particular group of antibiotics, one of the possible approaches involves immunization with a generic hapten. To our knowledge, this approach has not been used before for the class of aminoglycoside antibiotics. Neamin is such a generic structure present in a number of aminoglycosides. It is identical to a two-ring fragment of neomycin, and in shape and charge, it resembles parts of kanamycin and gentamicin.

Competition ELISA experiments performed with different coating conjugates, including BSA−neamin, showed that the anti-neamin antiserum could be used as a generic antibody preparation (i.e., showed desired displacement with various aminoglycoside residues) only when heterologous coating conjugates were used, i.e, with neomycin or kanamycin coupled to BSA by the glutaraldehyde method. With these coating conjugates, broad specific recognition of kanamycin, gentamicin, neomycin, and the hapten (neamin) could be obtained. A heterologous ELISA refers to a system in which the haptens used for the immunogen and for the (coating) conjugate have different chemical structures. In most cases, the haptens differ only slightly in structure. The usefulness of heterologous conjugates was reported earlier for the detection of various haptens, mostly pesticides (*19*, *20*−*22*). With respect to the class of aminoglycosides, Kitagawa and colleagues (*23*) reported a kanamycin ELISA that recognized the whole group of kanamycin analogues (kanamycin, debikacin, benakanamycin, amikacin, and tobramycin) in buffer by using an anti-kanamycin antiserum in combination with a heterologous hapten−enzyme label (tobramycin−galactosidase).

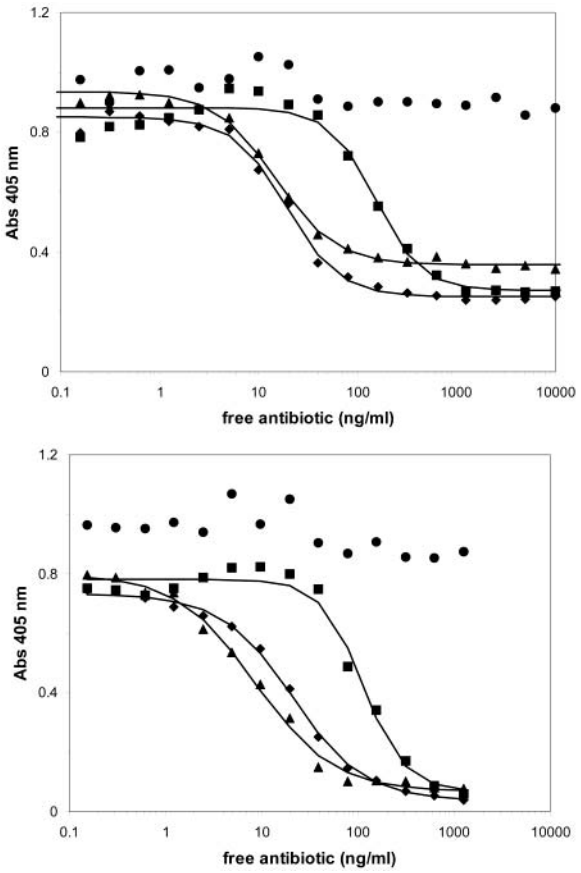

Figure 4. ELISA calibration curves obtained with the purified final anti-neamin serum from a KLH–neamin ACC-immunized rabbit SN329 on BSA–kanamycin (GDA 8.5) -coated plates in buffer (upper panel) and in undiluted raw milk (lower panel) with the following aminoglycosides as competitors: neomycin, ■; DH-streptomycin, ●; kanamycin, ▲; gentamicin, ◆. Data represent means from duplicate wells.

Table 1. Concentrations at 50% Inhibition (IC50) in the Competition ELISA Using the Protein G Purified Final Anti-Neamin Serum and a BSA–Kanamycin (GDA 8.5) Coating Conjugate

free aminoglycloside	matrix	anti-neamin Pab serum SN329 (protein G purified) IC50 (ng mL^{-1})
gentamicin	buffer	12
(MRL = 100 ng/mL)	raw milk	9
neomycin	buffer	121
(MRL = 500 ng/mL)	raw milk	113
kanamycin	buffer	17
(MRL = 150 ng/mL)	raw milk	21

It can be deduced that the specific antibodies induced by an immunogen conjugate might bind a homologous coating conjugate with such a high affinity that competition with free hapten in ELISA is impossible or visible only with amounts far above the MRL of the hapten. A heterologous conjugate generally will have a lower affinity for the hapten, and consequently, the free hapten will be able to displace the antibodies more sensitively. A good indication that differences in affinity did play a role in this case is the fact that lower dilutions of anti-neamin serum were necessary to obtain normal OD levels when heterologous coating conjugates are used.

Table 2. Quantitative Evaluation by the Charm II Aminoglycosides Test and the Competition ELISA (with Purified Serum of Rabbit SN329) of Naturally Contaminated Milk Samples with Neomycin

		concentration of neomycin (ng mL^{-1})	
sample	date of sampling	Charm II[a]	ELISA[b]
040036-1	29–06–1998	300	293.8 ± 9.5
040036-2	29–06–1998	300	317.8 ± 3.7
040036-3	30–06–1998	100	194.6 ± 0.6
040036-4	30–06–1998	100	177.8 ± 14.8
040036-5	01–07–1998	70	122.8 ± 4.8
040036-6	01–07–1998	70	106.2 ± 6.6

[a] Testing performed shortly after collection of the milk samples and storage at 4 °C. [b] Testing performed 1 year after collection of the milk samples and storage at –20 °C. Corrected for 1:1 dilution and quantified using the neomycin calibration curve shown in **Figure 4**.

In general, we have demonstrated that part of the hapten displacement problem in ELISA can be solved by the following practical adjustments and/or adapted strategies: using heterologous coating conjugates in competitive immunoassays (as described in this article); changing the coupling chemistry; or coupling to other functional groups when present on the hapten, yielding conjugates having the hapten exposed in different orientations.

Surprisingly, the bleed of day 66 and the final (purified) bleed showed different characteristics with the various heterologous coating conjugates. The antibodies from serum of day 66 (after the third booster) showed more sensitive displacement results in combination with the neomycin conjugate (GDA 7.3) as compared to the kanamycin conjugate (GDA 8.5). The situation was reversed for the final serum (after the 9th booster). A sensitivity change affecting all aminoglycosides was observed as well; compared with the use of the purified final bleed, the bleed of day 66 was 400 times more sensitive in detecting neomycin using the BSA−neomycin (GDA 7.3) coating conjugate. Affinity differences could play a role in this as well, because of maturation/adaptation of the immune response upon prolonged booster injections.

Polyclonal sera consist of a wide variety of antibody molecules of different specificity and affinity. Upon injection of the immunogen, the immune response might change, and each time an animal is bled, a different "cocktail" of such antibodies might be obtained. Therefore, optimal assay conditions and/or assay results can change between different boosters (*21*).

The overall results clearly show that the neamin hapten can be used to produce antibodies that cross-react with different aminoglycoside antibiotics such as gentamicin, kanamycin, and neomycin. The cross-reactivity of the aminoglycosides indicates that the presence of the central 2-deoxystreptamine ring is a prerequisite for recognition. DH-streptomycin, which contains a streptidine ring, is not at all or only weakly recognized, as expected considering the structure of neamin. The extent of cross-reactivity seems to depend on the position of the substitution on the deoxystreptamine nucleus (1,2- for neomycin and 1,3- for kanamycin and gentamicin), although the particular type of heterologous coating conjugate also plays a role in this respect. The competitive activity of streptomycin and several other aminoglycosides, such as sisomycin, tobramycin, and amikacin, was not tested. The aminoglycosides containing the 2-deoxy streptamine ring, the so-called subclass of deoxystreptamin antibiotics (e.g., sisomycin, tobramycin, and amikacin), will probably show cross-reactivity with the anti-neamin serum as well. Because of its close similarity in structure with DH-streptomycin, it is expected that streptomycin as well as the members of the subclass of streptidine antibiotics will not show significant cross-reactivity.

The broad specificity of the antiserum in combination with the use of a heterologous coating conjugate resulted in the development of an ELISA that easily detects neomycin, gentamicin, and kanamycin far below current MRL levels. The change to the real matrix, i.e., milk, did not have a dramatic effect on the sensitivity of the assay, as shown in **Table 1**. In addition, sample pretreatment can be completely omitted, resulting in a reduced assay time in comparison to most other ELISAs for the detection of aminoglycoside antibiotics in milk (*5*, *6*, *8*). Testing of acquired milk samples obtained after antibiotic treatment of a cow showed that the results are comparable for both the Charm II Aminoglycosides test and the competition ELISA when the concentration in the sample is 300 ng mL^{-1} of neomycin (MRL for neomycin in milk is 500 ng mL^{-1}). Surprisingly, for the milk samples containing neomycin concentrations of 100 ng mL^{-1} and lower, according to the Charm II Aminoglycosides test, the concentrations determined by ELISA were significantly higher (34−95%). The time interval between the neomycin determination in the competition ELISA and the Charm II test and, therefore, the storage conditions of the milk samples could play a role in this difference. HPLC measurements as a reference could have provided more insight. Possibly, the samples acquired contain substances that co-react in ELISA. Neomycin-like structures, not active in the receptor test, could be present and could bind to the anti-neamin antibodies. It is not expected that penicillin and amoxicillin, although present in the antibiotic cocktail that the cow received, would interfere; the structures of these β-lactam antibiotics deviate too much from the aminoglycoside antibiotic neomycin structure (see also **Figure 2**, lower panel: 6-aminopenicillanic acid). In addition, upon testing amoxicillin and cloxacillin in buffer up to 40 μg mL^{-1}, no competition was observed (results not shown).

In conclusion, milk samples contaminated with either gentamicin, kanamycin and/or neomycin can be positively assigned (i.e., their MRLs can be measured) due to the broad specificity characteristics of the antiserum SN329. Detection of kanamycin in milk at MRL level is particularly interesting because the Charm II Aminoglycosides test fails in this case, i.e., the limit of determination is around 3000 ng mL^{-1}, although a recently developed microbial inhibition assay exactly meets the MRL level of this residue, i.e., 150 μg kg^{-1} (*2*).

We have shown that the neamin hapten is useful for the production of an antiserum that cross-reacts with multiple aminoglycoside antibiotics such as neomycin, gentamicin, and kanamycin. However, the use of heterologous coating conjugates was necessary to develop a generic ELISA with sensitive detection of the subclass of deoxystreptamin antibiotics within the class of aminoglycoside antibiotics in undiluted raw milk at MRL levels. To the best of our knowledge, this is the first time a competition ELISA suitable for the screening of milk samples contaminated with neomycin, gentamicin, or kanamycin has been described.

ABBREVIATIONS USED

MRL, maximum residue level; EIA, enzyme immunoassay; DH-streptomycin, dihydro-streptomycin; BSA, bovine serum albumin; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; KLH, keyhole limpet hemocyanin; NHS, *N*-hydroxysuccinimide; ACC method, activated carbodiimide coupling method; GDA,

glutaraldehyde; IC50, concentration at 50% inhibition; PBS, phosphate buffered saline; MES, 2-[morpholino]-ethanesulfonic acid; DMF, *N*,*N*-dimethyl formamide; cFA, complete Freund's adjuvant; iFA, incomplete Freund's adjuvant.

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