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Development of a class-specific polyclonal antibody-based indirect competitive ELISA for detecting fluoroquinolone residues in milk*

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Abstract: Modified 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) method was employed to synthesize the artificial antigen of norfloxacin (NOR), and New Zealand rabbits were used to produce anti-NOR polyclonal antibody (pAb). Based on the checkerboard titration, an indirect competitive enzyme-linked immunosorbent assay (icELISA) standard curve was established. This assay was sensitive and had a working range from 0.12 to 68.40 ng/ml, with the half maximal inhibitory concentration (IC_{50}) and limit of detection (LOD) values of 2.7 ng/ml and 0.06 ng/ml, respectively. The produced pAb exhibited high cross-reactivity to fluoroquinolones (FQs) tested, and the IC_{50} values to enoxacin, ciprofloxacin, and pefloxacin were 3.1, 3.4, and 4.1 ng/ml, respectively. It also indicated that the concentrations of NaOH and methanol in assay buffer should not be higher than 10% and 30%. When spiked in milk at 5, 20, and 50 ng/ml, the recoveries for NOR, enoxacin, ciprofloxacin, and pefloxacin ranged 90.5%–98.0%, 84.0%–95.2%, 94.0%–106.0%, and 89.5%–100.0%, respectively. The results suggest that this class-specific pAb-based icELISA could be utilized for the primary screening of FQ residues in animal-original products.

Key words: Norfloxacin, Fluoroquinolones, Indirect competitive ELISA, Class-specificity, Milk
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1 Introduction

Infectious diseases are a serious problem for the livestock and poultry industries; therefore, various antibiotics and synthetic antibacterials are widely used for prevention and treatment. Among these, quinolones and fluoroquinolones (FQs) are the most important groups of synthetic antimicrobials. The original quinolones have only modest activity against Enterobacteriaceae and other Gram-negative bacteria. FQs are derived from the quinolone nalidixic acids by introduction of the piperazine moiety at Position 7 and a fluorine atom at Position 6 (Fig. 1), which are comparatively more effective in broad-spectrum activity and extensive tissue distribution than quinolone antibiotics (Zhang L. *et al*., 2011).

FQs have found widespread application in agriculture and aquaculture, and their use has resulted in the potential presence of these compound residues in foodstuffs of animal origin. In parallel to the exposure to low levels of these compounds, an increase of resistant human pathogens constituting a public health hazard, primarily through the increased risk of treatment failures, has been observed (Huet *et al*., 2006). In order to monitor FQ residue levels in foodstuffs, simple and economical methods are required. Traditionally, FQ residue analysis has relied upon

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high performance liquid chromatography (HPLC) (Hassouan *et al*., 2007; Christodoulou *et al*., 2008), liquid chromatography-mass spectrometry (LC-MS) (Delepine *et al*., 1998; San Martín *et al*., 2007), LC-MS/MS (Dufresne *et al*., 2007; Hermo *et al*., 2008), and other confirmatory methods. In general, chromatographic techniques require highly skilled personnel, laborious sample pretreatment, and expensive equipment, whereas immunoassay has been proven to be a rapid, cost-effective, and sensitive method, which is increasingly considered as an alternative method for routine monitoring.

In recent years, various immunoassay methods have been designed for detection of individual (Lu *et al*., 2006; Sheng *et al*., 2009) or generic FQs (Huet *et al*., 2006; Wang *et al*., 2007; Zhu *et al*., 2008; Huang *et al*., 2010; Zhang L. *et al*., 2011) in several matrices. However, the most cost-effective way of screening for veterinary residues is to develop immunoassays capable of measuring multiple targets in a single or generic test. This programme involves an initial, broad-spectrum surveillance system for a class of target compounds, followed by physico-chemical spectrometry techniques.

For the development of an FQ generic enzyme-linked immunosorbent assay (ELISA), the class-specific antibody recognition site should involve the piperazine ring common to all these drugs while specificity is determined by targeting areas of the molecule distal or space structure. In this article, we chose norfloxacin (NOR) to produce polyclonal antiserum and for subsequent immunoassay of different FQs, because the molecule structure of NOR (Fig. 1) closely mimics the common moiety in the FQs. We have therefore developed the specific ELISA standard curves for NOR, ciprofloxacin, pefloxacin, and enoxacin. Limited performance data for each assay in milk are also presented.

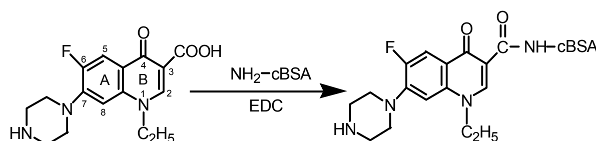

Fig. 1 Synthesis process of norfloxacin (NOR) immunogen through 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) method
cBSA: cationized bovine serum albumin

2 Materials and methods

2.1 Chemicals and materials

NOR, ciprofloxacin, pefloxacin, and enoxacin were purchased from Sigma (St. Louis, MO, USA), while other FQs were provided by the China Institute of Veterinary Drug Control (Beijing, China). Goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (GaRIgG-HRP) was purchased from Sino-American Biotechnology Company (Shanghai, China). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), Freund's complete adjuvant (FCA), and Freund's incomplete adjuvant (FIA) were obtained from Pierce (USA). *N*-hydroxysuccinimide (NHS) was obtained from Sigma-Aldrich (USA). Bovine serum albumin (BSA) and ovoalbumin (OVA) were supplied by Sigma while dialysis bag (8000–14000 Da) was from Solarbio Company (Beijing, China). Transparent 96-well polystyrene microtitre plates (Boyang Experimental Equipment Factory, Jiangsu, China) were used for the absorbance measurement. 3,3,5,5-Tetramethylbenzidine (TMB), phenacetin, and urea peroxide were purchased from Sigma Company (USA). A spectrophotometric microtitre reader (Multiskan MK3, Thermo Company, USA), provided with a 450-nm filter, was used for absorbance determination.

2.2 Synthesis of artificial antigen for NOR

2.2.1 Preparation of cationized BSA and OVA

In this procedure, carboxylic acid groups of BSA and OVA were converted into primary amine groups with an excess of ethylenediamine (EDA) (Zhang L. *et al*., 2011). A solution of 800 mg of BSA (or 300 mg of OVA) and 60 mg of EDC in 20 ml of phosphate buffered saline (PBS, 0.01 mol/L, pH 7.4) was added slowly into a solution of 18 mg of EDA in 20 ml of PBS while stirring. The mixture was incubated for 2 h at room temperature and then dialyzed against PBS while stirring to remove the free EDA. The solution was lyophilized, and the cationized BSA (or OVA) was defined as cBSA (or cOVA).

2.2.2 Preparation of immunogen for NOR

A modified EDC method was followed to synthesize the immunogen of NOR-cBSA (Zhang H.T. *et al*., 2011), and the procedure is presented in Fig. 1.

Thirty-five mg of NOR was suspended in 3 ml of dimethylformamide (DMF) and then 12 mg of NHS and 38 mg of EDC were added. During the following 24 h incubation in a dark chamber, the mixture was stirred at 37 °C. To this solution, 66 mg of cBSA, dissolved in 5 ml of PBS, was added dropwise while stirring. The resulting mixture was stirred by rotor in a dark chamber at 37 °C for 1 h, and then incubated with a reciprocal shaker for 3 h. After centrifugation at 3000×*g* for 10 min, the obtained supernatant was dialyzed against PBS for 4 d. When the absorption peak of the dialyzed solution disappeared, the immunogen of NOR-cBSA was stored in an ampoule at −20 °C.

2.2.3 Preparation of coating antigen for NOR

To synthesize the NOR-cOVA conjugate, a mixed anhydride technique was employed, slightly modified from Jiang *et al*. (2011b). Twenty mg of NOR was dissolved in 2 ml of DMF and 10 μl of triethylamine was added. During the following 1 h incubation in an ice bath, 30 μl isobutyl chloroformate was added and the mixture was stirred for another 1 h. Subsequently the resulting mixture was added dropwise to 40 mg of cOVA dissolved in PBS and DMF, which was then incubated at 4 °C for 6 h. The reaction mixture was dialyzed while stirring against PBS for 4 d to remove the uncoupled hapten. After lyophilization, the obtained NOR-cOVA coating antigen was stored at −20 °C (Fig. 2).

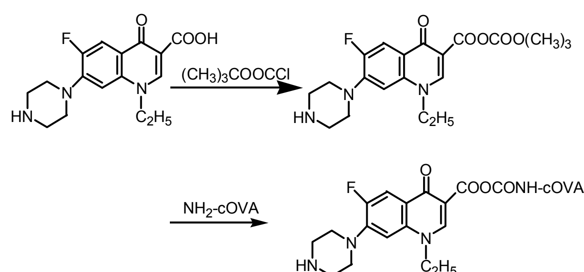

Fig. 2 Synthesis procedure for norfloxacin (NOR)-coating antigen through the mixed-anhydride method

2.3 Production of anti-NOR polyclonal antibody

Two Female New Zealand white rabbits were subcutaneously immunized at multiple sites in the back with NOR-cBSA conjugate. The initial injection was performed with 0.5 mg of conjugate in 0.5 ml of PBS plus 0.5 ml of FCA, and four subsequent boost injections were performed at three-week intervals with FIA as an emulsifying agent. Ten days after the final boost, two rabbits were euthanized by exsanguination and the whole blood sample was coagulated overnight at 4 °C, then centrifuged to separate the serum at 6000×*g* for 10 min. The crude serum was purified using a saturated ammonium sulfate (SAS) precipitation method, and the characteristics of the antibody were identified by ELISA. Sodium azide was added to the purified serum as a preservative at 0.02% (w/w), and the polyclonal antibody was then aliquoted and stored at −70 °C.

2.4 Antibody titer determination by indirect ELISA

Bidimensional titration assays were used to determine the coating antigen and the primary antibody concentrations, resulting in the following optimized protocol. Coating antigen of NOR-cOVA was appropriately diluted in carbonate buffer saline (CBS) and 100 μl was added to each well of the 96-well microtitre plates. After 2 h incubation at 37 °C, the plates were washed with PBS Tween-20 (PBST) three times and blocked with 250 μl/well of blocking buffer, followed by incubation for 1 h at 37 °C. After washing, the antisera (50 μl/well) serially diluted with PBS (from 1:100 dilution) were added across the plate. The plate was incubated for 15 min at 37 °C followed by washing as described above. Then GaRIgG-HRP (1:1000, v/v) was added, followed by incubation for 25 min at 37 °C. Plates were washed again and 60 μl/well of TMB substrate solution was added, followed by incubation for 15 min at room temperature. The enzymatic reaction was stopped with sulfuric acid (2 mol/L, 100 μl/well) and the yellow plate was spectrophotometrically read in a single wavelength at 450 nm. Preimmune serum and PBST were used as a negative control and blank control, respectively, and all data were measured in triplicate. Antibody titer was defined as the reciprocal of the dilution that resulted in an absorbance value of twice the background (Huang *et al*., 2011).

2.5 Development of indirect competitive ELISA standard curves

Competitive inhibition curves against NOR were established and the general assay procedures were described previously (Liu *et al*., 2007; Wu *et al*.,

2010). Based on the optimized concentrations, an indirect competitive ELISA (icELISA) method was developed, and the calibration curve was fitted based on the average of three separate assays in triplicate. The optical density (OD_{450}) of B_0 wells (containing all components except the FQ competitor) represents 100% activity, and the test wells (those containing working standards or samples) were normalized to the B_0 wells. Sensitivity was calculated using the IC_{50} values, which represented the concentration of NOR that produced 50% inhibition of antibody binding to the hapten conjugate. The working range for the icELISA was calculated as the concentration of the analyte providing a 20%–80% inhibition rate (IC_{20}–IC_{80} values) of the maximum signal. The limit of detection (LOD) was defined as the lowest analyte concentration that exhibits a signal of 15% inhibition (Jiang *et al*., 2011a). Specificity was defined as the ability of structurally related chemicals to bind to the antibody and cross-reactivity (CR) was calculated as: (IC_{50} of NOR)/(IC_{50} of FQs)×100%. The lower the cross-reactivity, the higher the specificity of NOR polyclonal antibody (pAb).

2.6 Chemical effects on assay performance

Immunoassay performance may be affected by chemical parameters such as ionic strength, pH values, organic solvent concentration, and sample matrices. The effects of these parameters were estimated by running standard curves under various conditions. The maximum absorbance (A_{max}, the absorbance value at zero concentration of analyte) and IC_{50} value were calculated, and the maximal A_{max}/IC_{50} ratio was chosen. In our study, NaOH and methanol were often added to the assay buffer to improve the solubility of the analyte. In order to evaluate the effects of these two detergents, NOR was diluted in assay buffer with 2%, 5%, 10%, 20%, or 30% NaOH, or with varying methanol concentrations (10%, 20%, 30%, 40%, or 50%).

2.7 Spiking tests in milk samples

2.7.1 Sample preparation

Some freshly collected milk samples with known backgrounds and certified as free of FQs (determined by HPLC) were used as blank samples in this study. Milk samples were vigorously shaken for 5 min, and then centrifuged at 4 °C with a speed of 8000×*g* for 10 min. After the floated fat was discarded, the rest of the milk was transferred to a calibrated flask, and diluted by a factor of 10× with the assay buffer before they were applied to establish the icELISA standard curves. FQs were prepared as concentrated solutions in assay buffer and kept at −20 °C in amber glass vials. To develop the competitive curves, the stock solution was serially diluted in milk, always using borosilicate glass tubes.

2.7.2 Precision and accuracy

Precision detection of the analytical procedure was determined by spiking a pool of negative milk samples with three different FQ concentrations (low 5 ng/ml, medium 20 ng/ml, high 50 ng/ml). To measure the intra-assay values, triplicate spiked milk samples were analyzed on six different days, while the inter-assay was determined from triplicate runs of six samples on a single day. Precision was expressed as the relative standard deviation (RSD) and accuracy was expressed as the recovery data of the spiked concentrations.

3 Results and discussion

3.1 Hapten conjugation

As a small molecule, NOR (M_W=361.14 (<1000) Da) (Fig. 1) has to be conjugated with a carrier protein in order to stimulate the immune response of rabbits and produce anti-NOR pAb. Among carrier proteins, BSA and OVA are two of the most often used, and they usually give satisfying results. However, under high concentrations, the drawbacks of poor solubility and protein cross-linking phenomenon are observed. BSA and OVA were cationized with an excess of EDA as described by Zhang L. *et al*. (2011), to convert carboxylic acid groups on the carrier proteins to primary amine groups. The use of modified carriers has the advantage that more amino groups on the carriers become available for coupling and that protein cross-linking is minimized. Moreover, the use of cationized carrier proteins treated with EDA is known to generate more immune responses as compared to their native forms (Lu *et al*., 2006).

3.2 Artificial antigen synthesis

In preparing class-specificity antibodies against veterinary drugs such as FQs, the key step is to design and synthesize the optimum artificial antigen. Our objective was to produce pAb that could recognize most of FQs' analogues, and to develop an icELISA standard curve that could determine the total array of FQs found in food-original samples. It has been proven that one or more FQs can be used as a hapten to synthesize the artificial antigen and produce class-specific antibodies, and we selected NOR as a desirable hapten based on the following factors. The FQ structures all possess a nitrogen-containing six-membered heterocyclic aromatic ring, a fluorine atom at Position 6 of ring A, a carboxylic acid group at Position 3, and a ketone group at Position 4 of ring B. Many of FQs have a piperazinyl ring at Position 7 of ring B. All of these common structural features would be helpful in obtaining class-specific pAb. In the NOR molecule, there is no substituent groups present on the piperazinyl ring (Fig. 1), and the molecule structure closely mimics the common moiety in the FQs. Therefore, NOR was selected to synthesize the artificial antigen and was evaluated for its ability to bind to other FQs.

3.3 Application of heterologous immunoassay

It has been proven that heterologous format is a useful strategy for the improvement of assay sensitivity in immunoassays (Jiang *et al.*, 2011b), which has also been demonstrated in our following experimental data. Heterologous systems in competitive ELISA are termed to indicate the differences in hapten structure, linker attachment site, bridge length, or conjugation method, which can effectively avoid the bridge interference, improve detection sensitivity and antibody specificity. In general, there are three situations to be considered: heterologous carrier protein, different conjugation method, and coupling locus heterogeneity. In the present study, we performed different conjugation procedures with the improved two-step EDC method to synthesize the NOR-cBSA immunogen and used mixed anhydride technology to prepare the NOR-cOVA coating antigen, achieving heterogeneous detection. In this way, a slight heterology (bridge character) was introduced inside the spacer arm and between the carboxyl group in Position 3 of NOR molecule and the carrier protein (Figs. 1 and 2). Overall, the described synthetic procedure constitutes a new strategy for the direct and simple synthesis of artificial antigen for FQs.

3.4 Establishment of icELISA standard curve

Rabbits injected with NOR-cBSA immunogen both produced the final antisera exhibiting high titer values after the fifth injection (fourth boost), therefore, their sera were mixed together for the following selection experiments, using the indirect ELISA and icELISA. It is well known that working concentrations of antibody and immobilized antigen are crucial factors to enhance the sensitivity in antigen-coated immunoassays. Checkerboard titrations were performed to determine the optimized combination between antibody and NOR-cOVA providing the highest sensitivity and the appropriate absorbance values (Table 1).

The optimal reagent concentrations were determined when the maximum absorbance (A_{max}) was between 1.5 and 2.0, and the dose-response curve of

Table 1 Determining parameters of coating antigen and NOR pAb by titration method

Coating concentration (μg/ml)	OD_{450}									
	Dilution fold of NOR pAb (×10^3)								Negative control	Blank control
	1	2	4	8	16	32	64	128		
10	2.942	2.615	2.284	2.195	1.983	1.746	1.614	1.489	0.125	0.097
8	2.802	2.586	2.345	1.990	1.869	1.655	1.512	1.427	0.096	0.084
6	2.691	2.387	2.166	1.923	1.822	1.621	1.451	1.328	0.182	0.071
4	2.446	2.246	2.007	1.858	1.672	1.469	1.313	1.245	0.126	0.083
2	2.215	1.988	1.712	1.594	1.527	1.351	1.218	1.094	0.175	0.102
1	1.883	1.726	1.548	1.356	1.284	1.151	0.948	0.868	0.181	0.085
0.5	1.529	1.441	1.332	1.145	1.165	0.991	0.746	0.694	0.131	0.094

inhibition ratio versus the NOR pursued the lowest IC_{50} values. According to the data, an increase in the concentrations of the antibody and NOR-cOVA enhanced the signal of the test, but resulted in decreased sensitivity of the method. On the other hand, inadequate reagents give low signals and may cause less accurate results. Therefore, optimal concentrations of the coated NOR-cOVA and anti-NOR pAb were determined to be 2.0 and 0.4 μg/ml in 1:16000 (v/v) dilution, respectively.

Fig. 3 shows the representative standard curve at the optimized assay conditions. NOR concentration values were calculated by interpolation from the calibration curve, which was based on the average of three separate assays, each run in triplicate. The LOD (also called the least detectable dose), turned out to be 0.06 ng/ml. Taking into account that NOR concentration is usually diluted ten times during sample preparation, the LOD value may be corrected as 0.6 ng/ml, still far from the negative cut-off point defined by the European Union (Huet *et al*., 2006) and the Ministry of Agriculture of the People's Republic of China (No. 278, May 22, 2003), which suggest an action range of 30–1500 μg/kg for certain veterinary medicines in foodstuffs of animal origin. The working range (also called dynamic range) for icELISA, were between 0.12 and 68.40 ng/ml, while the sensitivity (IC_{50}) of the assay was determined to be 2.7 ng/ml, suggesting that the developed immunoassay could allow the quantitative detection of NOR in matrix.

3.5 NaOH and methanol effects on assay

NaOH and methanol may interfere with antigen-antibody binding and increase the solubility of analytes, and were tested for their effects on the icELISA. Fig. 4 shows the normalized dose-response curves at various solvent concentrations. First, the influence of NaOH from 2% to 30% (v/v) in PBS on the immunosorbent assay was studied (Fig. 4a). Increasing the concentration of NaOH generally decreased and then increased the IC_{50} value, but with the incubation time increasing for color development. As the contents of NaOH increased from 2% to 10%, the absorbance gradually approached that of the PBS buffer. The average B_0 values for dilutions of 20% and 30% had absorbances of 1.463 and 1.219, respectively, as compared to 1.598 in PBS containing 10% of NaOH, indicating that 10% of NaOH allowed a significant gain in the detectability of this analyte. Taking into account these results, no reproducible inhibition curve could be observed, but only when the concentration of the solvent was less than 10%. Therefore, to accurately determine the NOR values, the NaOH solvent in the assay should be minimized.

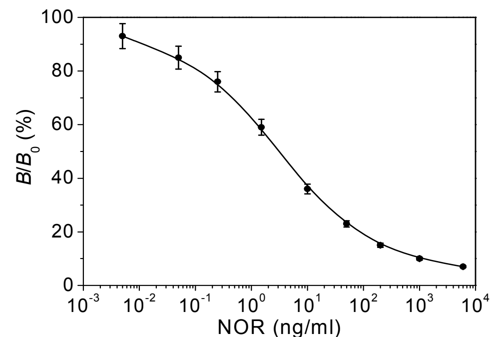

Fig. 3 Optimized standard icELISA inhibition curve for NOR

NOR-cOVA (2.0 μg/ml) as coating antigen was prepared in CBS (pH 9.6), anti-NOR pAb was diluted 1:16000 in PBS (pH 7.4), NOR was prepared in PBS containing 20% methanol, and GaRIgG-HRP was diluted 1:1000 in incubation buffer. Values are expressed as mean±SD (n=3)

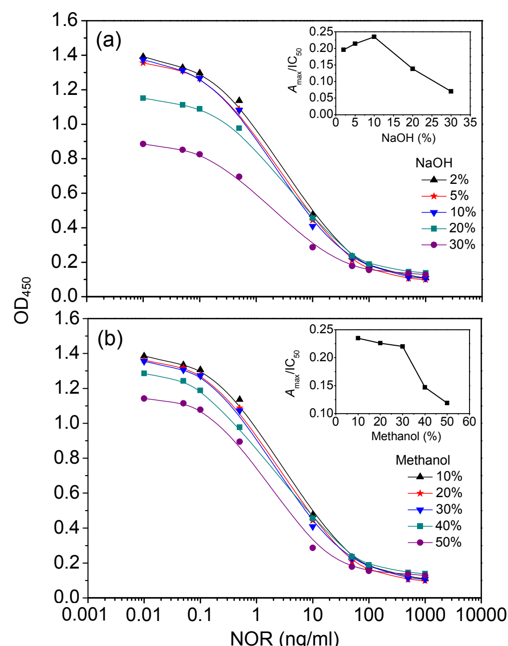

Fig. 4 Effects of NaOH (a) and methanol (b) concentrations on the icELISA inhibition curve

Insets indicate the fluctuations of A_{max}/IC_{50} as a function of solvent concentration. Each value represents the mean of three replicates

Fig. 4b shows the normalized dose response curves at different methanol compositions. When methanol concentration increased from 10% to 20%, the IC_{50} value improved from 2.8 to 2.7 ng/ml. This sensitivity improvement may be due to the dispersion and weakening of the nonspecific binding derived from pAbs. Concentrations of methanol higher than 30% resulted in lower absorbance and sensitivity drop as the higher methanol may weaken the antibody-hapten interaction. Therefore, 20% of methanol in PBS was the preferred assay buffer, and was employed for the remainder of this study. In practice, methanol concentration in matrix samples can be adjusted by simple dilution with concentrated buffer or PBS. If higher solvent concentrations are necessary in some assays, up to 30% of methanol could also be used because the sensitivity decrease is acceptable at this concentration.

3.6 Class-specific characteristics

Specificity is a phenomenon inherent to all immunoassays, which was evaluated by determination of the cross-reactivity based on the IC_{50} values of individual chemicals. In this study, our aim was to produce class-specific antibodies and develop an icELISA standard curve capable of detecting most of the FQ analogues. The closer structure of a hapten to that of the target analyte, the higher affinity of the antibody to target analytes and thus the higher sensitivity of the immunoassay. Therefore, we expected NOR to be ideal as an immunizing hapten and to produce a suitable antibody for multiresidue analysis, because it is a close mimic of the target molecule retaining the common functional groups of the FQs family. Investigations on cross-reactivity of the obtained antibody were undertaken by adding various competitors to compete with binding of the antibody to the coating antigen. Analytes that do not react with the antibody would produce absorbance near 100%; conversely, analytes that do react with the antibody would decrease in percentage of absorbance. The IC_{50} value and cross-reactivity rate for each compound are presented in Table 2. The values represent the mean of three separate experiments each of which contained a minimum of three replicates.

Of all the cross-reacting analogues, the produced antibody would react with the chemicals containing a comparable structure, and thus this assay exhibited the highest cross-reactivity to enoxacin (87.1%), ciprofloxacin (79.4%), and pefloxacin (65.9%). In our research, the immunogen was synthesized by the linkage of carboxylic acid group of NOR with the amino group of the carrier protein (Fig. 1). This design exposes the piperazinyl ring, the common part for FQs drugs as the immunodominant area, which is also the furthest group of NOR from the linking point. Consequently, the high similarity of structures of substitutents (Positions 1 and 7) between these four chemicals may explain their high-reactivity. In the following experiments, we also selected the detection analytes of NOR, enoxacin, ciprofloxacin, and pefloxacin to establish the icELISA standard curves in milk.

Table 2 Cross-reactivities of FQs analogues in the NOR immunoassay

Analogues	IC_{50} (ng/ml)	Cross-reactivity (%)
Norfloxacin	2.7	100.0
Enoxacin	3.1	87.1
Ciprofloxacin	3.4	79.4
Pefloxacin	4.1	65.9
Enrofloxacin	9.0	30.0
Lomefloxacin	11.6	23.3
Sarafloxacin	15.4	17.5
Amifloxacin	18.8	14.4
Ofloxacin	24.1	11.2

Although enrofloxacin has the same piperazinyl moiety at Position 7, the extra ethyl group located in the piperazinyl ring and cyclopropyl ring in Position 1 may be responsible for the different electronic structures. Thus, medium cross-reactivity (30%) is not surprising. The combined effects of changing the group at Position 1 and at the piperazinyl ring can be evaluated by comparing the IC_{50} value (2.7 ng/ml) for NOR to the IC_{50} values of lomefloxacin (11.6 ng/ml), sarafloxacin (15.4 ng/ml), and amifloxacin (18.8 ng/ml) (Table 2). The spatial structure of the target compounds seems to play an important role in the interaction between the antigen and antibody in this case. As to ofloxacin, it lacks the ethyl group at Position 1 of NOR, but has some heteroatom structures. A comparison of the IC_{50} values demonstrates that the loss of this group and substitution with other groups has diverse degrees of effects on binding.

It is deduced that the substitution group on the basic structure of FQs plays an important role in

epitope determination, and the results also reveal the importance of the spatial volume of the group at Positions 1 and 7 in NOR for antibody recognition. The aromatic ring as well as the electronegative fluorine would be expected to involve a number of binding interactions. Generally, class-specificity of anti-NOR pAb proves that this immunoassay has the potential to be incorporated into a multiresidue programme for simultaneously detecting nine kinds of veterinary FQs residues in animal-producing foodstuffs.

3.7 Multiresidue standard curves for FQs in milk

In food samples, chemical compounds such as pigment, salt, sugar, fat, and others might affect the binding of antibodies and analytes, which could reduce the precision and accuracy of the results. Therefore, removing the matrix effects is important in the ELISA study. In general, dilution of sample extracts and preparation of standard curves in the blank tissue are two effective ways to minimize the interference of matrices. Thus, ELISA runs with the standard FQs dissolved in milk extracts were applied in the present study.

For use in the ELISA performance evaluation, known negative samples spiked with FQs at various concentrations were extracted and diluted 10-fold in assay buffer before application onto the 96-well microtiter plates. Fig. 5 shows the standard inhibition curves of FQs dissolved in milk extracts. Under 10-fold dilution conditions, the icELISA calibration curves were obtained and the measurement parameters were calculated for NOR (y=−9.8752ln(x)+39.832, IC_{50}=2.8 ng/ml), enoxacin (y=−9.4216ln(x)+38.649, IC_{50}=3.3 ng/ml), ciprofloxacin (y=−8.9125ln(x)+38.246, IC_{50}=3.7 ng/ml), and pefloxacin (y=−7.8865ln(x)+37.892, IC_{50}=4.6 ng/ml). As shown in Table 2, the IC_{50} values for FQs obtained in milk were a little higher than that obtained in PBS buffer (2.7 ng/ml).

3.8 Analysis of spiked samples

The analytical performance of ELISAs is commonly assessed by spiking milk samples with the target analytes. Each spiked sample was evaluated three times in duplicate to verify the accuracy of the developed icELISA, and the results are presented in Table 3. The NOR spiked at 5, 20, and 50 ng/ml obtained an average recovery rate of 98.0%, 90.5%, and 91.6% for intra-assay and 94.0%, 93.0%, and 92.4% for inter-assay, respectively. The RSDs ranged from 6.9% to 13.8% for intra-assay and 8.2% to 12.8% for

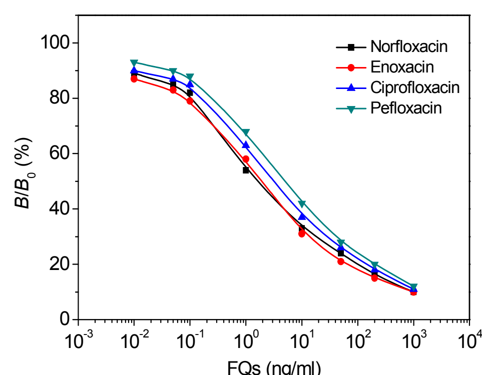

Fig. 5 Multiresidue determination icELISA standard curves using class-specific NOR pAb for FQs

Table 3 Intra- and inter-assay precisions and accuracies of samples tested

Sample	Spiked (ng/ml)	Intra-assay			Inter-assay		
		Found (ng/ml)	Recovery (%)	RSD (%)	Found (ng/ml)	Recovery (%)	RSD (%)
Norfloxacin	5	4.9±0.5	98.0	10.2	4.7±0.6	94.0	12.8
	20	18.1±2.5	90.5	13.8	18.6±1.9	93.0	10.2
	50	45.8±3.2	91.6	6.9	46.2±3.8	92.4	8.2
Enoxacin	5	4.2±0.3	84.0	7.1	4.6±0.6	92.0	13.1
	20	17.3±1.9	86.5	10.9	18.9±1.7	94.5	8.9
	50	45.5±3.8	91.0	8.4	47.6±2.8	95.2	5.9
Ciprofloxacin	5	5.2±0.5	104.0	9.6	5.3±0.7	106.0	13.2
	20	19.5±1.8	97.5	9.2	18.8±1.9	94.0	10.1
	50	48.2±1.9	96.4	3.9	47.5±2.5	95.0	5.3
Pefloxacin	5	4.6±0.6	92.0	13.0	5.0±0.6	100.0	12.0
	20	17.9±1.5	89.5	8.4	18.2±1.7	91.0	9.3
	50	47.6±2.6	95.2	5.5	47.9±2.9	95.8	6.1

inter-assay. Regarding the enoxacin, repeated analyses showed good precision. The mean RSD values ranged from 5.9% to 13.1%, only slightly affected by the concentration, and the recoveries were in the range of 84.0% to 95.2%, thus indicating reasonable precision and accuracy. The intra- and inter-day assays showed relatively good accuracy for ciprofloxacin, while the precision of the higher concentration was superior to that of the lower concentration, in which RSDs ranged from 3.9% to 13.2%. With respect to pefloxacin, intra-assay RSDs were from 5.5% to 13.0%, and the equivalent figures (from 6.1% to 12.0%) were found in inter-assay. The recoveries were all above 89.5%, demonstrating a reasonable level of accuracy. These results demonstrate that the values obtained are acceptable; the sample extraction methods and the icELISA developed for the analysis of FQ residues in milk are satisfactory.

4 Conclusions

In this study, we have prepared a high-quality polyclonal antibody with high cross-reactivity to nine FQs tested for the first time. The feasibility to apply this antibody in a competitive ELISA to detect the residues of FQs has been explored, and the proposed method is inexpensive, accurate, fast, and simple. Under the 10-fold dilution of milk, the respective icELISA standard curves for NOR, enoxacin, ciprofloxacin, and pefloxacin were established, and the recoveries of intra-assays (84%–104%) and inter-assays (91%–106%) for FQs were satisfactory. It can be concluded that this generic ELISA is capable of detecting simultaneously nine FQs in milk, and may potentially be applied to the analysis of FQs in other matrices, such as biological fluids. As the generic assay cannot identify individual drugs or distinguish the components of a mixture, a noncompliant result would require a confirmatory method, such as the LC-MS/MS assay.

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