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Multiresidue Detection of Fluoroquinolones: Specificity Engineering of a Recombinant Antibody with Oligonucleotide-Directed Mutagenesis

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ABSTRACT: Screening of a group of antibiotics from foodstuffs has traditionally relied on sophisticated chemical or physical analysis methods, such as liquid chromatography and mass spectrometric applications. The equipment for these techniques is expensive and not always applicable for high throughput screening. There is a need for an easy and cost efficient detection method for simultaneous screening of structurally similar compounds. Here we describe the engineering of a recombinant antibody which was subjected to oligonucleotide targeted random mutagenesis to emphasize the generic specificity of fluoroquinolone binding. Phage display together with small sized fluoroquinolone derivatives was used to find antibodies of high affinity and generic specificity. The most improved antibody was used to develop a time-resolved fluorescence immunoassay which was further optimized and applied for the detection of fluoroquinolone residues from spiked whole milk samples. The assay can be used to efficiently screen all European Agency for the Evaluation of Medicinal Products (EMEA) controlled fluoroquinolones from whole milk samples with detection levels ranging from 0.2 to 68 $\mu g\ L^{-1}$.

KEYWORDS: *fluoroquinolones, time-resolved fluorescence immunoassay, milk*

INTRODUCTION

Fluoroquinolone (FQ) antibiotics are a group of broad spectrum antibiotic agents widely used to treat various bacterial infections. The pharmacological effect of FQs is based on the inhibition of replication in both Gram-negative and Gram-positive bacteria via interaction with DNA−gyrase enzyme involved in DNA replication. Quinolone structure prevents the religation of DNA strands by stabilizing the cleaved form of gyrase−DNA complex.[1] A wide variety of fluoroquinolone derivatives with different pharmacological properties have been synthesized,[2] and over 30 of them are used as veterinary and human therapeutics.

The excess use of FQs in domestic animals has led to the emergence of FQ-resistant bacterial strains and side effects in humans, which has brought a need for more extensive monitoring of food products by public health agencies. The European Agency for the Evaluation of Medicinal Products (EMEA) in Europe, the Food and Drug Administration in United States, and the Chinese Ministry of Agriculture has set maximum residue limits (MRLs) for monitoring the levels of FQs in animal-originated foodstuffs. Several analytical techniques have been applied to detect FQs from foodstuffs and animal tissues. The conventional methods to detect FQs include liquid chromatographic combined with fluorescence, ultraviolet[3] or mass spectrometric[4] detection. However, these methods are quite time-consuming and require expensive equipment, and the widespread use of structurally variable FQs in livestock and aquacultures calls for more efficient detection methods. The use of antibodies as a basis for the assay can facilitate the development of rapid assays suitable for high throughput screening. Different immunoanalytical approaches have indeed been developed ranging from detection of a specific FQ[5] to multianalyte assays,[6] taking typically advantage of generic antibodies, i.e., antibodies with the capability to recognize several members among a group of chemicals.[7,8] Although multiresidue immunoassays which use antibodies for detection of FQs can be based on both polyclonal[8,9] and monoclonal antibodies,[10,11] the latter ones are preferable reagents as the risk of depletion of the antibody is not an issue. Antibody engineering is a useful tool for the improvement of different properties of antibodies, such as affinity,[12] stability,[13] or specificity.[14] The goal of this study was to further enhance the properties of a broad-specificity antibody and apply it to an optimized TRF-immunoassay for the multiresidue detection of FQs from whole milk. We have previously shown that with the use of antibody engineering it is possible to emphasize the generic binding properties of an antibody.[15] With the help of oligonucleotide-directed mutagenesis and phage display, we modified the binding characteristics of a previously described recombinant antibody to develop a binder suitable for high throughput screening assay of FQs.

EXPERIMENTAL SECTION

Materials and Reagents. All microbiological reagents were made as described in Sambrook et al.[16] Streptavidin (SA) and rabbit antimouse (RAM) coated microtiter plates were purchased from Innotrac diagnostics (Finland). DELFIA series instrumentation used in immunoassays: plate washer and Victor 1420 fluorometer were from Perkin-Elmer (Turku, Finland). The solutions: assay buffer (AB), wash buffer, enhancement solutions were from Kaivogen (Turku, Finland). Enzymes used for cloning and mutagenesis reactions were from New England Biolabs (USA). Fluoroquinolones: difloxacin (DIF), sarafloxacin (SAR), ciprofloxacin (CIP), danofloxacin (DAN), enrofloxacin (ENR), norfloxacin (NOR), flumequine (FLU), and marbo-

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floxacin (MAR) were purchased from Sigma-Aldrich (>99% pure). The FQs were initially dissolved in 30 mM sodium hydroxide at 5 mg L^{-1} from where standard solutions (100 μg L^{-1}) for the assays were diluted in AB and stored at +4 °C. Degenerate oligonucleotides were designed to be used in Kunkel mutagenesis. Oligonucleotides were purchased from Thermo Scientific (USA). Labeled FQ derivatives were used as described previously in Leivo et al.[15] Streptavidin-coated magnetic nanoparticles and magnetic bead concentrator were purchased from Dynal (Norway). The freshly milked (<16 h) whole milk was obtained from a local farmer.

CDR-H3 Library Construction. An antibody library was constructed on the basis of a previously developed FQ antibody,[15] which was used as a template for further mutagenesis reactions. Stop mutations and *SacII* restriction sites were introduced to the area of mutagenesis before construction of the libraries. For template production, 5 mL of K12-CJ236 cells (OD_{600} = 0.5) were infected with 1×10^{10} tfu/mL of m81516 phages for 30 min at +37 °C without shaking. The volume of the culture was diluted to 40 mL (SB), and the cells were grown until the OD_{600} was ∼0.5 before superinfection with VCSM13 helper phages (Stratagene, USA). Phage propagation was continued overnight at +26 °C, 300 rpm. Phages were precipitated twice with polyethyleneglycol/natriumchloride (PEG/NaCl) before isolation of the single-stranded DNA (ssDNA) with the QIAprep Spin M13 kit (QIAgen, USA) according to the manufacturer's protocol. Purity and concentration of the ssDNA were confirmed by gel electrophoresis. The oligonucleotides used to generate stop mutations and *SacII* sites to the complementarity determining regions (CDR) were phosphorylated with T4 PNK enzyme (NEB) for 60 min at +37 °C. Annealing of 50 pM of phosphorylated oligonucleotides with 1.2 μg of dUssDNA template was done by heating the reaction to +90 °C for 2 min and gradually cooling it to +4 °C. Synthesis of the dsDNA was done in T4 DNA ligase buffer supplemented with 25 mM dNTPs, 3 WU T4 DNA ligase, and 15 U T7 DNA polymerase. The reaction was carried out for +16 h at 20 °C before purification with the QIAgen PCR purification kit. The DNA was transformed to electrocompetent XL1-Blue cells with a Bio-Rad genepulser. Successful mutagenesis was confirmed from sequence data and digestion analysis. The main library Lib-H3 was constructed similarly where degenerate oligonucleotides and uridinylated-ssDNA concentrations in the synthesis reaction were 150 ng and 20 μg, respectively. Transformation was done in four aliquots to SS320 cells for each library. Plasmid DNA was isolated with the QIAgen Spin Miniprep kit and treated with *SacII* restriction endonuclease for background elimination before transformation to XL1-Blue cells. Fab-phages were rescued from 100 mL cultures containing 0.25 mM isopropyl β-D-thiogalactopyranoside (IPTG, Promega) for promoter induction.

Antibody Selections. Selections were done with 1×10^6 MyOne-C1 streptavidin-coated magnetic beads (Dynal, Norway). Three selection rounds were done for the constructed library with the use of biotinylated FQs. FQs used in this study included biotinylated sarafloxacin (bio-SAR), norfloxacin (bio-NOR), and core-FQ (bio-cFQ).[15] The amount of biotinylated antigens was 10 ng initially, and it was reduced by 50% after each round. Calculating the enrichment factor for each selection round was used to monitor the enrichment of the specific Fab-phages. SA beads without biotinylated antigens were used to control the enrichment of unspecific or background binders. The phages used for selection were incubated in SA-coated wells for 60 min with slow shaking at +21 °C prior to antigen binding. Binding of biotinylated antigen with the Fab phages was done in TBT-0.1 buffer (50 mM Tris, 150 mM NaCl, 1% BSA-fraction V, 0.1% Tween-20, pH 7.5) for 2 h with rotamix at room temperature. SA-coated MyOne beads (1×10^6) were added to collect the antigen−Fab−phage complex. The reaction was continued for 30 min with rotamix at room temperature. The beads were harvested with magnetic bead concentrator (MPC, Dynal) and washed three times with TBT-0.5 buffer. Specific Fab phages were eluted with 0.1 M glycine-HCl (pH 2.2) for 30 min at room temperature. The beads were collected, and the eluate was transferred to a tube containing 2 M Tris-HCl (pH 9.0) for neutralization. XL1-Blue cells (1 mL) were infected with the eluate before plating. The LA plates were incubated 16 h at +37 °C. The phage stock was prepared by harvesting the cells from the plates and inoculating 20 mL of SB medium with 1×10^7 cells. The culture was incubated at +37 °C with 300 rpm shaking until the OD600 reached 0.5. VCSM13 helper phages (Stratagene, USA) were used to superinfect the cells for 30 min at +37 °C. The culture was supplemented with 75 mg L^{-1} of kanamycin and 0.1 mM IPTG, and phage propagations was continued for 16 h with 250 rpm shaking at +26 °C. Phages were purified with polyethyleneglycol-NaCl precipitation. The precipitation step was repeated three times before storing the phages in TSA/BSA (50 mM Tris, 150 mM NaCl, 0.02% NaN_3, 1% BSA, pH 7.75) solution at +4 °C.

Antibody Expression. Colonies from the output plate of the selection round three (92 clones) were inoculated to a 96-well tissue culture plate (Sarstedt, USA) containing 170 μL of SB. The cultures were incubated for 6 h in 700 rpm shaking at +37 °C. The Fab production was induced with 0.1 mM IPTG, and the plate was transferred to 700 rpm shaking at +26 °C for 16 h incubation. The cells were collected with 15 min centrifugation 4000 rpm, at +4 °C, and the supernatant was used for immunoassay to screen antigen-recognizing antibodies. Twelve mutants were cloned into the pAK400 vector, and antibody production was carried out as described earlier.[15] The antibody was extracted from the periplasmic space with lysis buffer (50 mM Tris pH 8, 1 mM EDTA, and 0.2 mg mL^{-1} lysozyme) for 30 min at +21 °C. The antibody used in the milk sample immunoassays was cultured in 50 mL of SB. Antibody production and lysis was done as described previously.[15] Purification was done with the His Spintrap kit according to manufacturer's protocol (GE Healthcare, USA).

Immunoassays. All immunoassay steps were done in 100 μL at room temperature with shaking for 1 h unless otherwise described. The immunoassays for the sorting and analysis of the libraries and found mutants were done in 200 μL. The enrichment of the libraries was measured with an immunoassay where the serial dilutions of phage stocks were incubated in an antigen-saturated microtiter well. Time-resolved fluorescence was measured from europium-labeled anti-fd phage IgG (150 μg L^{-1}), which was used as a tracer antibody. Signal to background ratio (S/B) was calculated from the average counts per second (CPS) in relation to the label background. Screening was focused in finding of an antibody with different binding properties to marbofloxacin with a one-point inhibition assay. The supernatant from each clone in the 96-well plate culture was transferred into two separate RAM coated wells with 1:20 dilution in AB. Europium-labeled human serum albumin conjugated sarafloxacin (Eu-cHSA-SAR, 75 μg L^{-1}) was used as a tracer. Inhibition percent was calculated from the signal to background ratio. Antibodies which showed altered binding properties to MAR from the screening were characterized with competitive immunoassay. The antibodies were attached to a RAM plate with 1:10 dilution, and IC50 values were measured from five different concentration points (10−5000 μg L^{-1}) of MAR. Time-resolved fluorescence (TRF) europium signal was measured and the IC50 values were determined with the use of Sigmaplot 12 (Systat software Inc.) based on the equation f = min + (max-min)/(1 + $10^{(\log EC50-x)}$). The limit of detection (LOD) was determined from the standard inhibition curves by subtracting standard deviation (SD, multiplied by 3) from the mean signal of eight FQ free samples. The linear range of the assay was set between the concentrations ranging from IC20-IC80.

The complete characterization of target FQs was done for the best mutant found from the H3 mutagenized library (mH3D7). The pH of the assay buffer used in the immunoassay was optimized, since some of the FQs are sensitive to pH variation.[19] The pH of TBS buffer (50 mM Tris, 150 mM NaCl, pH 9.0) was adjusted to pH values ranging from 5.0 to 9.0 with HCl (10 M). For the immunoassay, RAM wells were coated with 50 ng of mH3D7 antibody and incubated 1 h at +21 °C. The IC50 value in each pH was determined from a six-point inhibition curve where concentrations between 1 and 5000 μg L^{-1} tracer (eu-cHSA-SAR 75 μg L^{-1}) were used in a competitive immunoassay. The cross-reactivity immunoassay was done similar to pH optimization with the exception of antigen concentrations which ranged from 0.05 to 10000 μg L^{-1}. The measurement and data analysis

Table 1. Detected Amino Acid Changes in the Found Mutant Antibodies and Respective IC50 Values for MAR[a]

	CDR-H3															
clone	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	IC50 MAR (μg L^{-1})
m81516	R	P	I	Y	D	G	Y	Y	G	E	I	F	F	D	W	511
mH3A1								N	R							539
mH3A11						S	E	S								819
mH3B1					S		N		R							718
mH3D7						Y				Q						200
mH3D9					Y		F	D		G		Y				1083
mH3G1					Y	S										382

[a]Amino acid numbering according to IMGT.[18]

were done as described previously. The immunoassay performance with food samples was tested by spiking fresh raw milk samples (n = 3) with FQs at concentrations (0.5−100 μg L^{-1}). The samples were incubated 30 min at +21 °C with rotation and diluted 1:10 in assay buffer before measurement. The recoveries were calculated from a six-point standard inhibition curve of concentrations between 0.1 and 1000 μg L^{-1}. The standard curve assay buffer was supplemented with 1:10 of milk to eliminate the effect of the sample matrix. The pH of the assay buffer was set to 6.7 for the cross-reactivity and assay performance measurements.

RESULTS AND DISCUSSION

Library Construction and Sorting. Oligonucleotide-directed mutagenesis was used to randomize the CDR loop of the antibody m81516, which was previously developed in our laboratory.[15] The mutations were introduced in the heavy chain CDR-H3 loop with the use of degenerate oligonucleotides since the CDR-H3 loop has a prominent role in the interaction with antigen.[17] The CDR-H3 library was targeted for soft randomization with three nucleotide changes in the CDR loop consisting of 15 amino acids (IMGT[18]). The mutagenesis libraries were constructed in the phagemid vector pAK200/pIX to enable display of the Fab fragments on the surface of the M13 filamentous phage as a fusion to phage pIX coat protein.[19] The strongest enrichment was observed from the selections done with bio-SAR (data not shown). Consecutively the 92 colonies selected for the screening were picked from the output plates of the last selection round. The screening of the mutant antibodies was done with an inhibition assay consisting of only MAR and eu-cHSA-SAR, since all the other FQs were detected with abundant affinity.

Characterization. After selections, the six antibodies were cloned into the pAK400 plasmid for more efficient expression of soluble Fab. The clones were sequenced and their IC50 values were determined by a time-resolved fluorescence-based immunoassay. The clones were found to have 2−5 amino acid changes in the CDR-H3 region (Table 1). The mutants with improved binding characteristics had a high prevalence of tyrosine. This might be due to the phenolic side-chains interaction with the planar structure of the FQs. The most improved mutant antibody (mH3D7) was selected for closer study. The antibody has two amino acid changes in the CDR-H3 hypervariable loop, G104Y and E108Q. The two mutations had a major effect on the binding of FLU and MAR and only a slight effect on the other FQs (Table 2). The cross-reactivity profile was evaluated with eight EMEA monitored FQs, used in a competitive TRF assay. The IC50 values for the target FQs are below the MRLs set for bovine milk by EMEA (Table 2). On the basis of the cross-reactivity profile of the antibody, the antigens can be divided in three groups (Table 2) by the side-group attached in position 1 of the 4-quinolone structure (Figure 1). The 4-fluorophenyl group (DIF, SAR) clearly has a positive effect on binding; antigens containing the group can be detected at very low concentrations. (0.22−0.24 μg L^{-1}) The majority of the controlled FQs (CIP, DAN, ENR, FLU, NOR), which contain a carbon-based side-chain in position 1, are recognized with a uniform pattern and concentrations ranging from 5 to 12 μg L^{-1}. In the structure of MAR, the region that distinguishes it from the other FQs are the nitrogen and oxygen atoms attached to the 4-quinolone structure. Either one or both of these atoms create a steric hindrance in the antibody−antigen interaction resulting in a 5−300 fold increase to the IC50 value in comparison to the other FQs.

Table 2. Optimized IC50 Values and Cross-Reactivity for the Mutant mH3D7[a]

	mH3D7		m81516		
FQ	IC50 (μg L^{-1})	CR (%)	IC50 (μg L^{-1})	CR (%)	MRL[b] (μg L^{-1})
DIF	0.22 (−0.09)	100.0	1.24	16.0	400
SAR	0.23 (−0.03)	92.5	0.20	100.0	100
CIP	5.03 (−5.26)	4.4	7.51	2.6	100/100
DAN	4.69 (−4.00)	4.7	19.57	1.0	30/200
ENR	4.78 (−1.47)	4.6	12.87	1.5	100/100
NOR	5.14 (−0.13)	4.3	10.44	1.9	N/A
FLU	12.68 (−4.23)	1.7	337.95	0.1	50/100
MAR	68.05 (−120.76)	0.3	549.58	0.1	75/150

[a]The improvement gained from the optimization of the assay is presented in parentheses. [b]Maximum residue limits for bovine milk/meat.

Assay Optimization and Performance. The assay conditions were optimized before further analysis. The most important parameter proved to be the assay buffer pH, which clearly has a major impact on the IC50 value of MAR (Figure 2). The same effect has been previously detected for DAN and FLU, and is likely due to the zwitterionic nature of the FQs.[20] Assay performance was tested with the use of spiked whole milk samples. The matrix effect to the immunoassay performance is shown in Table 3. The composition of whole milk creates some interference to the assay; however the performance is satisfactory even without sample pretreatment. Addition of methanol and precipitation of insoluble proteins with trichloroacetic acid was also tested, but it only had a slight effect on the assay performance (data not shown).

The majority of the FQs can be found with recoveries >70% (Table 4). The sample matrix seems to create some interference to the assay, which has an effect in the target recovery at higher FQ concentrations. For SAR and DIF, the higher fortification levels are outside the linear range of the

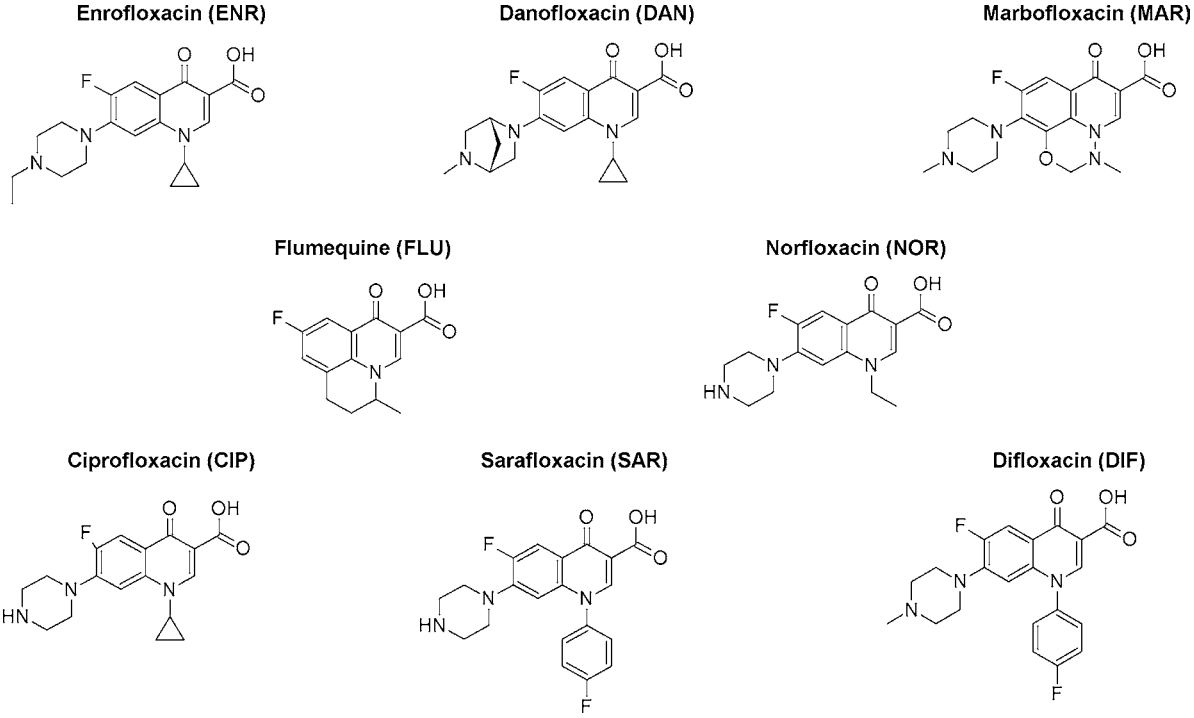

Figure 1. Fluoroquinolone antibiotics. The structures of the eight FQs controlled by EMEA.

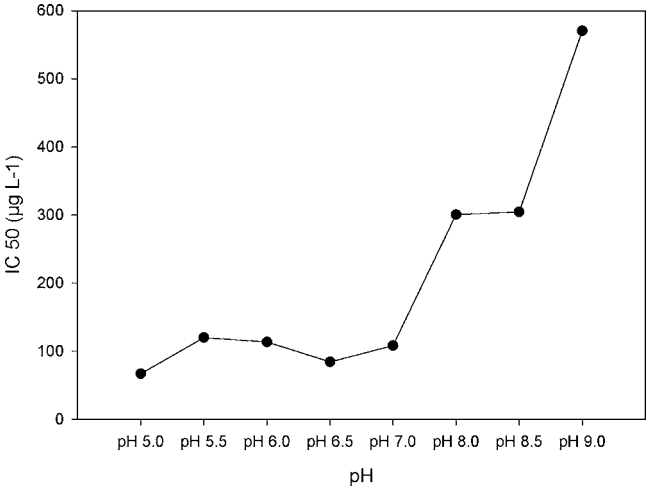

Figure 2. Assay buffer pH optimization.

Table 3. Analysis of the Assay Performance and Matrix Effect

	assay buffer		milk	
FQ	LOD ($\mu g\ L^{-1}$)	linear range ($\mu g\ L^{-1}$)	LOD ($\mu g\ L^{-1}$)	linear range ($\mu g\ L^{-1}$)
DIF	<0.01	0.1−4.72	0.09	0.1−0.85
SAR	<0.01	0.1−3.43	0.11	0.1−0.8
CIP	0.32	0.7−52.5	0.34	0.5−14.6
DAN	0.33	0.6−48.9	1.3	2.1−86.9
ENR	0.39	0.8−39.0	1.34	1.9−39.6
NOR	0.32	0.7−67.4	2.27	3.2−51.8
FLU	0.6	1.2−77.1	3.8	4.8−37.5
MAR	2.3	8.5−50.5	33.9	44−275

assay and the recoveries are lower as a result. These results may be because the samples were not treated before or after the spiking.

Table 4. Detection of MAR from Spiked (n = 3) Milk Samples

FQ	spiked ($\mu g\ L^{-1}$)	found ($\mu g\ L^{-1}$)	recovery (%)	CV (%)
SAR	0.5	0.39	77.5	8.21
	2	1.38	69.2	6.01
DIF	0.5	0.45	89.7	23.18
	2	1.31	65.4	4.29
CIP	5	4.58	91.6	5.82
	10	9.79	97.9	5.11
ENR	5	5.50	110.0	0.77
	10	9.16	91.6	1.06
DAN	5	3.62	72.3	4.11
	10	9.78	97.8	0.07
NOR	5	4.52	90.4	16.02
	10	10.09	100.9	0.57
FLU	10	9.43	94.3	1.14
	20	14.65	73.3	3.09
MAR	50	37.07	74.1	2.23
	100	78.82	78.8	0.01

We started with the mediocre monoclonal antibody 6H7, and in this and the previously described study[15] (Table 2) we show, by using antibody engineering, improved recognition of marbofloxacin over 4-fold (856.6 → 188.8 $\mu g\ L^{-1}$). Further improvement was gained from the optimization of the immunoassay which decreased the MAR IC50 value almost 3-fold (188.8 → 68.1 $\mu g\ L^{-1}$). With the use of oligonucleotide-directed mutagenesis, we have optimized a multiresidue immunoassay based on time-resolved fluorescence for screening of eight EMEA-controlled FQs in a single assay. The identification of individual FQs from the samples would require the use of a conventional liquid chromatography based methods. However, the LC-based methods are more time-consuming, require special instrumentation, and are dependent on sample pretreatment. The immunoassay described in this

study can be used to efficiently screen all EMEA-monitored FQ residues directly from raw milk samples.

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Notes

The authors declare no competing financial interest.

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