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Simultaneous Detection of Ofloxacin and Lomefloxacin in Milk by Visualized Microplate Array

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Abstract: A sensitive, simple, inexpensive, simultaneous analytical method for ofloxacin and lomefloxacin in milk by immunoassay without the need of time-consuming or complex pre-treatment steps was reported. A 96-well microplate was used as solid support, on which ofloxacin antigen and lomefloxacin antigen were immobilized, respectively. After immobilization, a mixture of relevant antibodies and standard solutions containing the analytes or samples were added to the array reaction area, then added silver nanoparticles (AgNPs) labeled secondary antibody. Silver enhancement technique was applied to amplify the detection signals, which produced black image on array spots visible with naked eyes. The signals were detected with a microarray scanner; therefore the analyte residues could detect quantitatively. The Limits of Detection (LOD) (3SD) were estimated to be 0.24 ng/mL (ofloxacin) and 0.35 ng/mL (lomefloxacin).

Keywords: Visual Detection, Protein Microarray, Silver Enhancement, Quinolones, Ofloxacin, Lomefloxacin

Introduction

Fluoroquinolones (FQs) show efficacy against a variety of bacterial diseases and are indicated in the treatment of local and systemic diseases caused by a wide range of gram-negative and gram-positive bacteria. Moreover, low cost and availability make them the most frequently use for the prevention and treatment of animal infection in animal husbandry.

It was important to ensure that quinolones were used appropriately. Residues of QNs could have harmful effects on human consumers, including allergic reactions, liver damage, and gastrointestinal disturbance. In addition, long-term exposure to such species can lead to an increased drug-resistance of microbial strains, and they also include a possible detrimental effect on the environment.

Accurate, sensitive and portable analytical methods for the determination of antibiotic residues in foodstuffs were demand. Several methods for QNs residues analyses in food have been developed. Some of them are based on microbiological assays as they are easy to perform. Unfortunately such methods are time-consuming (usually require 12-24 hours for microbe growth) and lack the needed specificity and sensitivity. Due to their high sensitivity and specificity, chromatographic techniques have been evaluated, including HPLC (Geng Nan Wang et al. 2015; Zhang et al. 2012; Zhang et al. 2015) and HPLC-fluorescence(Fan et al. 2015; Phonkeng and Burakham 2012; Rambla-Alegre et al. 2011; Yao et al. 2015; Zhang et al. 2017). Used as confirmed method, such as LC-MS (Aguilera-Luiz et al. 2013; Dorival-García et al. 2016; Junza et al. 2014; Lombardo-Agüí et al. 2012; Tian et al. 2016), could be used to separate the mixture, and detected by qualitative and quantitative. However, chromatographic methodologies required high-cost instrumentation and samples usually needed extensive and time-consuming processing, such as extraction and purification before the analysis, and usually had to be run by skilled technicians in a laboratory. Therefore, development of rapid, sensitive, and cost-effective methods for residues detection and screening in routine assays is still a research demand.

Immunochemical methods were gaining in confidence owing to their high sensitivity, simplicity, and cost-effective, which made them particularly useful in routine work. They were capable of detecting low concentrations of residues in many samples in a short time, and often did not require extraction or clean-up steps, which made these assays particularly suitable for screening evaluation. However, Colloidal Gold Immunoassay Strip (CGIS)(Byzova et al. 2014; Peng et al. 2017; Wang et al. 2017; Wu et al. 2016) detection speed was fast, usually about 10 minutes, but cannot be quantitative. Enzyme-Linked Immunosorbent Assay (ELISA) (Li et al. 2013; Tufa et al. 2015; Zhu et al. 2011), Fluorescence Immunoassay (FIA)(Leivo et al. 2011; Leivo et al. 2013), Chemiluminescence Enzyme Immunoassay (CEI)(Zeng et al. 2016), these method can be detected quantitatively and

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own high sensitivity, but not fast. Immunoaffinity Chromatography (Jiang et al. 2015; Sun et al. 2016), did not provide quantification of multiple analytes. Therefore, a rapid, sensitive, accurate, cost-effective and convenient method which can detect multiple antibiotics residues in milk remains an important need in food safety monitoring.

In this paper, antibiotics in milk without any extraction or clean-up was designed to be achieved. In addition, this method provided improved LOD and dynamic range in milk with a good repeatability.

Experimental

Materials and instruments

Quinolones (QNs) inclued Ofloxacin, Lomefloxacin, Norfloxacin, Fleroxacin, Enrofloxacin, Pefloxaein, Difloxacin, Ciprofloxacin, Sarafloxacin, Nalidixic acid, Danofloxacin, Enoxacin, Oxolinic acid, Flumequine, Pazufloxacin, Sparfloxacin, Gatifloxacin, Marbofloxacin, Orbifloxacin, and silver enhancement solution including solution A and solution B were all obtained from Sigma-Aldrich. NaCl, $Na_2HPO_4.12H_2O$, KH_2PO_4, KCl, Ethylene Di-amine Tetra Acetic Acid (EDTA), Tween-20, was purchased from Nanjing chemical reagent Co., Ltd. (Nanjing, China). Bovine Serum Albumin (BSA) was from Merck. Antibodies of ofloxacin and lomefloxacin, and goat anti-mouse IgG which was labeled AgNPs were kindly provided by Nanjing Xiangzhong biotechnology Co. Ltd. (Nanjing, China).

Pure water of 18.2 MΩ cm^{-1} was generated in-lab from a Milli-Q water system. All solutions were made by triple deionized water. Phosphate-Buffered Saline (PBS) (pH 7.4) consisted of 137 mM NaCl, 1.5 mM KH_2PO_4, 7 mM Na_2HPO_4, and 2.7 mM KCl was used as spot buffer and assay buffer. PBS containing 1mM EDTA (PBS- EDTA) was used as milk sample dilution solutions. The wash buffer consisted of PBS+0.05% (v/v) Tween-20. The blocking solution was contained 10 mg/mL BSA in PBS. All buffers were filtered through 0.22 μm pore size filter before used.

The microplate arrays were prepared by TMAR spotter (Tsinghua University, Beijing, China). Washing step was carried out using an automatic 96-well plate washer (BioTek Instruments, Inc. America). Thermoshaker used to shake the microplates, Microarray scanner (QARRAY 2000) performed data acquisition and clear flat-bottom 96-well plate were from Nanjing Xiangzhong Biotechnology Co. Ltd. (Nanjing, China).

Preparation of ofloxacin and lomefloxacin artificial antigens

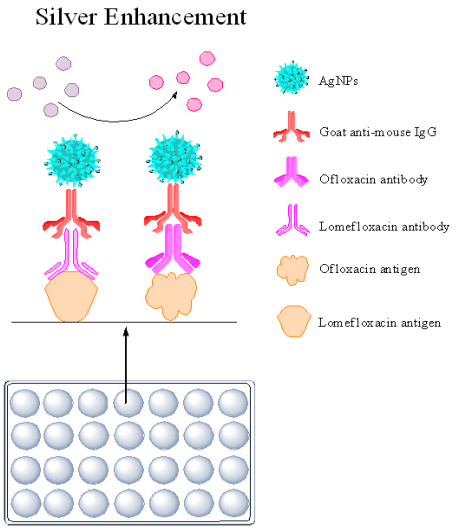
3.2 mg of ofloxacin (OFX) or lomefloxacin (LOF), 2.3 mg of N-hydroxysuccinimide (NHS), 3.8 mg of 1- (3- dimethylaminopropyl) -3-Ethylcarbodiimide hydrochloride (EDC) was weighed into 4 mL centrifuge tube, and then added 2mL DMF; After completely dissolved, the solution was transferred into 10 mL flask, then placed on a magnetic stirrer and wrapped in foil to ensure darkness. After stirring at room temperature for 24 hours, it was removed and placed into a 4 mL centrifuge tube which marked solution A. Solution A was slowly dropped into 6.5 mL of 1 mg/mL BSA, reacted in darkness for 12 hours. Dialyzed for 3 days in PBS buffer in 4°C, then sub-installed and stored in -20°C.

Microarray preparation

Artificial antigens (ofloxacin-BSA and lomefloxacin-BSA) were spotted into clear flat-bottom 96-well plate by spotter. Coating antigens which diluted by spot buffer were arrayed 10 nL/spot with a 500 μm spot-to-spot pitch in 1×6 array, each artificial antigen was in triplicate. After spotted, microarray was incubated at 37°C for 2 h. In this step, the coating antigens were immobilized into the clear flat-bottom 96-wells. After immobilization, microarray surface was treated with 200μL blocking solution at 37°C for 1 h, in order to minimize further unspecific bindings. After incubation, the microarray plate was washed with wash buffer which used an automated plate washer and then sealed in foil packets for storage at 2-8°C.

Principle and the procedure of indirect competitive microplate array immunoassay

Indirect competitive microplate array immunoassay principle was presented in Scheme 1. In a microarray immunoassay analysis the following experimental



Scheme 1: Schematic illustration of ofloxacin and lomefloxacin detected by visualized microarray immunoassay platform.

procedure was performed. The competition is established by the addition of a mixture of 25 µL the standard (or the sample), a known amount of 25 µL mixed antibodies and 25 µL AgNPs labeled goat anti-mouse IgG in a total volume of 75 µL/well. The plates were covered with adhesive sealers and incubated at 25°C for 30 min on a thermoshaker (shaking at 600 rpm). After that, the plates were washed 3 times with 10 mM PBST solution (300 µL/well). After the corresponding washing step, 50 µL silver enhancement solution including solution A and solution B was added to each well, the microplates were further incubated for 12 min at 37°C in dark. At the end of colorimetric reaction, after washing the plates following the previous procedure, each well was washed 3 times with 250 µL pure water.

Microarray imaging and data processing

The microplate array was imaged with microarray scanner (QARRAY 2000) and data acquisition used the corresponding software to quantify the signal on the sample spot area and expressed as Relative Light Units (RLUs). The calibration curve was presented by a linear relationship.

Cross reactivity calculation

The cross-reaction rate was generally judged using a competitive inhibition curve. Different concentrations of antigens and interference were used as competition inhibition curves to calculate their respective binding ratios (B/B0), and their respective concentrations at IC_{50} were calculated. Cross-reaction rates were calculated according to the following formulas (1).

$CR=(IC_{50}$ of analyte)/(IC_{50} of interference)×100% (1)

IC_{50} is necessary concentration of analyte or interference to induce a signal inhibition of 50%.

Samples preparation

Metal ions such as calcium, iron, magnesium and zinc in milk which should be considered that ofloxacin and lomefloxacin had a high possibility of forming chelation complex. Prior to analysis actual samples, milk solution was prepared with 1 mL milk in 9mL sample dilution solutions. Milk (pasteurized milk, UHT milk and Skimmed milk) was purchased from local supermarket in Nanjing, China. The milk was confirmed to be free of ofloxacin and lomefloxacin by HPLC-MS. A recovery test of OFX and LOF was carried out by adding blank milk samples with the different concentrations at levels of 1, 10, 100 ppb to evaluate the accuracy and precision of the methods.

Results and Discussion

Characterization of ofloxacin and lomefloxacin antigens

UV spectra of Ofloxacin, lomefloxacin and BSA was detected, but before and after coupled with BSA, UV absorption spectrum curve and λ_{max} were changed. When concentrations of ofloxacin and lomefloxacin were 0.01mol/L, concentration of BSA was 1mg/mL, UV absorption spectrum was shown in Figure 1, which could be found OFX-BSA and LOF-BSA absorption curve and λ_{max} were all changed. Ofloxacin λ_{max} was 302 nm, after coupled with BSA, λ_{max} was 286 nm. Lomefloxacin λ_{max} was 290 nm, after coupled with BSA, λ_{max} was 287nm.

Optimization concentrations of coating antigens and antibodies

Competitive immunoassay was performed by concentrations of coating antigens which were immobilized onto the plate, primary antibodies and second antibodies. Coating antigens of ofloxacin and lomefloxacin were 1:2, 1:4, 1:8, 1:16 dilution respectively; antibodies of ofloxacin and lomefloxacin were 1:25000, 1:50000, 1:100000, 1:150000 dilution respectively. The second antibodies were 1:100 dilution. Results can be seen in Figure 2. The choice of optimal conditions were done to obtain a reasonable signal (the Relative Light Units (RLUs) were about 45000). Optimal assay conditions were as follows: appropriate concentrations of ofloxacin-BSA and lomefloxacin-BSA were 1:4 dilution; best concentrations of antibodies of ofloxacin and lomefloxacin were 1:100000.

Cross-reactivity

Cross-reactivity between ofloxacin-BSA and lomefloxacin BSA;

The ofloxacin-BSA and lomefloxacin-BSA antigens 1:4 dilution were fixed to the bottom of the microplate with a spotted instrument. Ofloxacin and lomefloxacin antibody 1:100000 dilution were

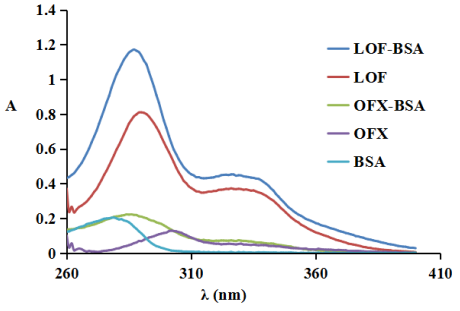


Figure 1: UV spectrum of ofloxacin (OFX), lomefloxacin (LOF), BSA, antigens

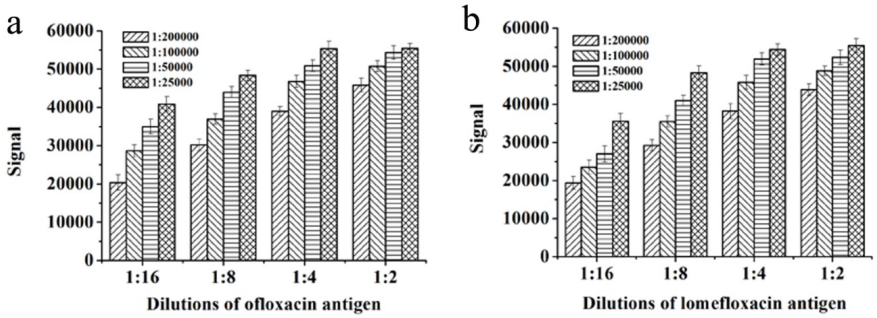


Figure 2: Coating antigens of ofloxacin-BSA and lomefloxacin-BSA were 1:2, 1:4, 1:8, 1:16 dilution respectively; antibodies of ofloxacin and lomefloxacin were 1:25000, 1:50000, 1:100000, 1:150000 dilution respectively. The second antibodies were 1:100 dilution.

added into the wells respectively. The experimental results are shown in Table 1. If there was needed to detect ofloxacin and lomefloxacin simultaneously, Ofloxacin-BSA and Lomefloxacin-BSA antigen and antibody cross-reaction rate should be less than 10% between each other. From the table we can see that cross-reaction rate of ofloxacin antibodies and lomefloxacin-BSA antigens, lomefloxacin antibodies and ofloxacin-BSA antigens were all less than 10%. Thus under the premise of high sensitivity, ofloxacin and lomefloxacin can be detected simultaneously in one well.

Cross-reactivity of antibodies ofloxacin and lomefloxacin;

Specificity of the antibody is evaluated by cross-reactivity study which is important part of evaluation the immunoassay. Study was performed using structure similar compounds of quinolones, similar compounds including: Ofloxacin, Lomefloxacin, Pefloxaein, Danofloxacin, Flumequine, Enoxacin, Norfloxacin, Marbofloxacin, Orbifloxacin, Ciprofloxacin, Naphthalene acid, Oxolinic acid, Fleroxacin, Sparfloxacin, Difloxacin, Pazufloxacin, Sarafloxacin. The cross-reactivity for each compound was given in Figure 3. These results demonstrated that ofloxacin antibodies cross-reactivity less than 10% except marbofloxacin, lomefloxacin antibodies cross-reactivity less than 10% except fleroxacin and marbofloxacin.

Cross-reactivity

To evaluate the parameter of this developed methods, calibration curves were prepared using serious concentration levels of each analyte. Figure 4 depicted the different calibration curves obtained the detection of ofloxacin and lomefloxacin in buffer. As a matter of fact, the competition occurs for all target molecules and the specific signal obtained on each probe decreases with the analyte concentration, as expected in a competitive immunoassay. The ofloxacin dynamic range is from 0.05 ng mL−1 to 12.8 ng mL−1. The calibration curve was calculated as: y=-0.3446x+0.4287, r=0.9849. The lomefloxacin dynamic range was from 0.05 ng mL^{-1} to 12.8 ng mL^{-1}. The calibration curve was calculated as: y=-0.3055x+0.4797, r=0.9927; in the calculation formula, y: B/B0%, x: lg C (ng/mL).

Table 1: Cross-reactivity between ofloxacin-BSA and lomefloxacin-BSA

Item/signal value	Ofloxacin-BSA		Lomefloxacin-BSA	
	Signal	Cross-reactivity	Signal	Cross-reactivity
Ofloxacin antibody	44234.67	100.0%	3483.87	7.9%
Lomefloxacin antibody	2609.73	6.1%	42818.05	100.0%

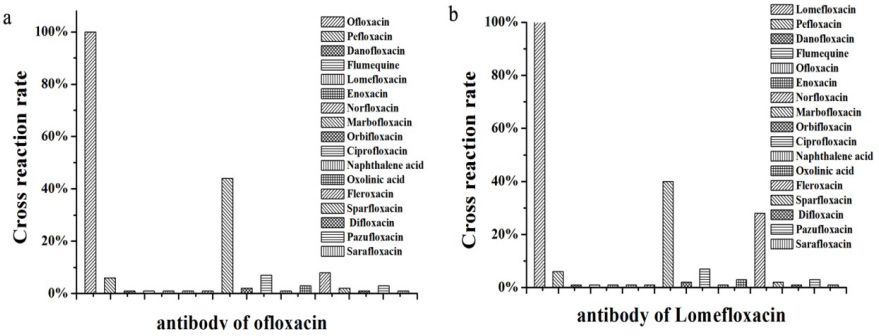


Figure 3: (a) Ofloxacin antibodies cross-reactivity with other quinolones similar compounds. (b) Lomefloxacin antibodies cross-reactivity with other quinolones similar compounds

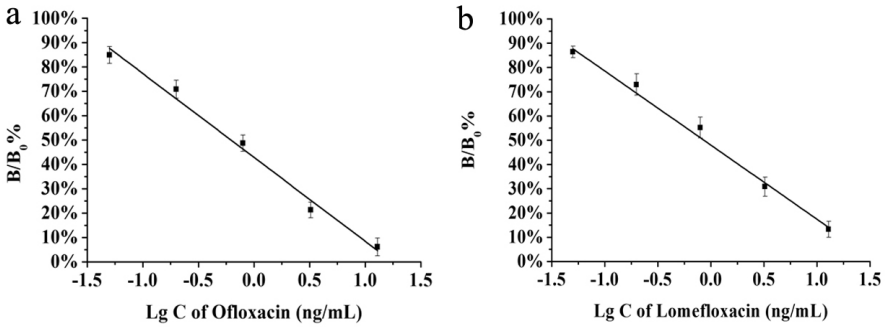


Figure 4: Calibration Curves of ofloxacin and lomefloxacin. Ofloxacin and lomefloxacin dynamic range were all from 0.05 ng mL^{-1} to 12.8 ng mL^{-1}. The calibration curve of ofloxacin was calculated as: y=-0.3446x+0.4287, r=0.9849. The calibration curve of lomefloxacin was calculated as: y=-0.3055x+0.4797, r=0.9927; In the calculation formula, y: B/B0%, x: lg C (ng/mL)

Recovery study

According with calibration curve to calculate the concentration of ofloxacin and lomefloxacin in milks which after a simple dilution (1 mL milk was diluted with 9 mL PBST-EDTA, pH 7.2). The milk was confirmed to be free of ofloxacin and lomefloxacin by HPLC-MS. Ofloxacin (0, 1 ng/mL, 10 ng/mL, 100 ng/mL) and lomefloxacin (0, 1ng/mL, 10ng/mL, 100ng/mL) were spiked in milk solution. The recovery and repeatability study were performed in three replicates and the results were quite satisfactory as shown in Figure 5 and Table 2.

Limits of Detection (LOD) of ofloxacin and lomefloxacin were 0.24ng/mL and 0.3 5ng/mL, respectively. Limits of Quantitation (LOQ) of ofloxacin and lomefloxacin were 0.32 ng/mL and 0.47 ng/mL, respectively. LOD is defined as the concentration corresponding to 3 standard deviations above the mean of blank samples and the LOQ as the concentration corresponding to 10 standard deviations above the mean of blank samples.

Figure 5: Picture of results of Ofloxacin and lomefloxacin detected by Visualized Microarray. From top to bottom, left to right was different concentrations of ofloxacin and lomefloxacin(0, 0.05ppb, 0.2ppb, 0.8ppb, 3.2ppb,12.8ppb), and milk sample which spiked different concentrations(0, 1ppb, 10ppb, 100ppb).Samples were repeated 2 wells

Table 2: The recoveries of different concentrations of OFX and LOF in milk

	Spiked concentration	Average (ppb)	Recovery	RSD
OFX	0	0. 2	-	5.9%
	1 ppb	1.2	120.0%	10.3%
	10 ppb	10.4	104.0%	11.1%
	100 ppb	87.5	87.5%	8.8%
LOF	0	0.3	100.0%	5.8%
	1 ppb	1.3	130.0%	8.4%
	10 ppb	10.8	108.0%	9.4%
	100 ppb	82.4	82.4%	4.2%

Conclusions

The microplate array proposed method, which combines a very simple sample treatment can be applied for a throughput screening of ofloxacin and lomefloxacin residues in milk samples and it is well suited for screening large number of samples. Unlike other ELISA assays, no treatment of the sample extract other than dilution was involved, even for complex samples. Thus, only 10-fold dilution is required to avoid the matrix effect. Moreover, the relevant cross-reactivity of the assay towards fluoroquinoles indicates higher recognition profile and sensitivity. A detection capability of 0.24 ng mL^{-1} and 0.35 ng mL^{-1} for ofloxacin and lomefloxacin in milk indicates that the method is very sensitive. All these, in addition to the robustness of the assay, makes this microplate array method very suitable for effective control of cross contamination during feed production or to detect unauthorized use of fluoriquinolones in bad farming practices.

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