Preparation of an Antidanofloxacin Monoclonal Antibody and Development of Immunoassays for Detecting Danofloxacin in Meat

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ABSTRACT: Danofloxacin (DAF), a third-generation fluoroquinolone (FQ), is widely used as a broad-spectrum antibacterial drug to prevent diseases in livestock and poultry. In this study, a highly specific and sensitive monoclonal antibody (mAb) against DAF was prepared. Also, the mAb was used for the indirect competitive enzyme-linked immunosorbent assay (icELISA) and immunochromatographic strip for the detection of DAF residues in meat. The IC50 of the icELISA based on this mAb was 1.39 ng/mL, and the limit of detection was 0.2 ng/mL. According to the cross-reactivity (CR) experiment, the ELISA that we developed was highly specific and had low CR with other FQ analogues. Moreover, the cut-off of the immunochromatographic strip developed for detecting DAF in meat was 5 ng/mL. Overall, the developed ELISA and immunochromatographic strip based on the prepared mAb were proved reliable for the rapid detection of DAF in meat and can be considered as effective screening methods for food safety and quality management.

1. INTRODUCTION

Danofloxacin (DAF), a broad-spectrum fluoroquinolone (FQ) antibacterial drug,1,2 is widely used to prevent and treat infectious diseases in animals.3,4 However, antimicrobial resistance,5,6 hemolytic-uremic syndrome,7 thromboembolism,8 and adverse effects on the central nervous system9 have been reported owing to DAF abuse. Therefore, some countries established maximum residue levels (MRLs) of DAF in different animal species and tissues. In the USA, only 200 ng/g of DAF residue in the liver or muscle of cattle is permitted by the Food and Drug Administration. In the EU, the MRLs of DAF are 200 ng/g for bovine muscle and 400 ng/g for liver or kidney.10 In China, the MRLs of DAF set by the Ministry of Agriculture (no. 278, 2003.5.22) are 200 ng/g for muscle, 100 ng/g for fat, 400 ng/g for liver, 400 ng/g for kidney, and 30 ng/g for milk in cattle and sheep.

Thus far, many instrumental analytical methods11–13 have been developed for the detection of DAF residues. These methods can quantitatively and qualitatively detect DAF and other veterinary drugs. However, these methods are limited by their need of highly trained personnel, bulky apparatus, and complicated preparation.14 Immunochemical methods are alternative approaches owing to their simplicity, rapid operation, and cost-effectiveness.15,16 Shanin et al.17 established a fluorescence polarization immunoassay and ELISA based on polyclonal antibodies, which exhibited a limit of detection (LOD) for DAF of 13 ng/mL. Liu et al.18 prepared an anti-DAF antibody using New Zealand white rabbits and developed an ELISA for DAF residue in chicken liver with an IC50 value of 2.0 ng/mL. Sheng et al.19 developed an ELISA based on polyclonal antibody using New Zealand white rabbits for the detection of DAF residue in beef, chicken, and pork muscles, which exhibited an IC50 value of 5.4 ng/mL. To the best of our knowledge, these studies were based on polyclonal antibodies obtained from New Zealand white rabbits, and no immunoassays based on murine monoclonal antibodies (mAb) have been reported. In contrast to polyclonal antibodies, mAb have the advantages of stable performance, high specificity, and high cost-efficiency in the long term. Therefore, in this study, a mAb against DAF was prepared, and an ELISA and immunochromatographic strip based on the prepared mAb were developed to detect DAF in meat.

2. MATERIALS AND METHOD

2.1. Reagents and Instruments. FQ standards including DAF, ofloxacin (OFL), moxifloxacin (MOX), ciprofloxacin (CIP), cinoxacin (CIN), sarafloxacin (SAR), fleroxacin (FLE), enrofloxacin (ENR), marbofloxacin (MAR), and pefloxacin (PEF) were purchased from J&K Scientific Ltd. (Shanghai, China). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysulfosuccinimide (NHS), Freund’s incomplete adjuvant, Freund’s complete adjuvant, bovine serum albumin (BSA), and ovalbumin (OVA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Horseradish peroxidase-labeled goat antimouse secondary antibody (HRP-sAb) was purchased from Univ-bio Co., Ltd. (Shanghai, China).

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used for cell fusion, such as HAT and HT solutions, were purchased from Thermo Fisher Scientific Inc. (Shanghai, China). Other reagents and chemicals were acquired from the National Pharmaceutical Group Chemical Reagent Co., Ltd. (Shanghai, China). The nitrocellulose (NC) membrane, absorbent pad, and sample pad were purchased from Millipore (Bedford, MA).

Eight-week-old female BALB/c mice were obtained from the Nanchang University Laboratory Animal Center (Nanchang, China).

A BioDot XYZ platform equipped with a motion controller was supplied by BioDot (Irvine, CA). A portable gold nanoparticle (GNP) reader (HELMEN) was purchased from Zhejiang Fenghang Science Instrument Co., Ltd. (Zhejiang, China). A high-speed freezing centrifuge was purchased from Xiang Yi (Hunan, China). A vacuum-drying oven was purchased from Shanghai Fumma Laboratory Instrument Co., Ltd. (Shanghai, China).

2.2. Preparation of the Immune and Coating Antigens. The immune antigen was prepared by coupling DAF to BSA using the active ester method, according to previous studies.20,21 The specific procedure was as follows: 1 mM DAF, 8 mM EDC, and 8 mM NHS were dissolved in 200 μL of 0.01 M phosphate buffered saline (PBS, pH = 7.4) and stirred at room temperature for 4 h in the dark. Then, the mixtures were added dropwise to 10 mM BSA in 0.01 M carbonate buffer solution (pH = 9.6) and stirred overnight. The danofloxacin—bovine serum albumin (DAF—BSA) was obtained after the above reactants were dialyzed for 3 days in 0.01 M PBS. The coating antigen danofloxacin—ovalbumin (DAF—OVA) was synthesized in the same way. The successful couple of the complete antigens was verified by an UV spectrophotometer.

2.3. Production of mAb. The mAb was obtained by immunizing healthy female BABL/C mice through hypodermic injection. To obtain high-quality antibodies, the mice were immunized multiple times at an interval of 3 weeks. For the first time, the immunization was performed with 100 μg/mouse immunogen (DAF—BSA) emulsified in Freund’s complete adjuvant. The immunization dose was changed to 50 μg/mouse immunogen (DAF—BSA) emulsified in Freund’s incomplete adjuvant in the next four immunizations. One week after the fifth immunization, the mice were tail-bleed, and the serum was evaluated by indirect competitive enzyme-linked immunosorbent assay (icELISA) to select a fusion mouse with the best affinity and inhibition for DAF. Three days before the fusion, the selected mouse was boosted with 25 μg of immune antigen mixed with saline. For the fusion, the selected mouse was killed, and its spleen was removed to fuse with SP2/0 myeloma cells at the exponential stage. The icELISA was performed for screening hybridoma cells with high affinity and inhibition after they were cultured in HAT solution for 4 days and in HT solution for 3 days. Subsequently, the selected hybridoma cells were cloned three times by limiting the dilution to form stable cell lines. The cloned hybridoma cells were injected into the abdomen of mice to produce ascites. Then, the ascitic fluid was purified by the saturated ammonium sulfate purification method to obtain mAb.

2.4. icELISA Process. The mAb was evaluated with icELISA, which was slightly modified compared with conventional icELISA. Briefly, a 96-well microplate was coated with 100 μL/well coating antigen DAF—OVA for 2 h. Then, the microplate was washed three times with washing solution and blocked with 200 μL/well blocking buffer for another 2 h. After removing the blocking solution and washing the plate three times, 50 μL of standards and 50 μL of mAb in 0.01 M PBS were added to react for 1 h. After washing, 100 μL/well of HRP-sAb (diluted 1:3000 with 0.01 M PBS) was added to react for 30 min. The excess HRP-sAb was then washed, and 100 μL/well of TMB solution was added to develop color changes. After 15 min, 50 μL of 2 M H₂SO₄ was added to terminate the reaction. The entire experiment was reacted in an incubator at 37 °C. Finally, the optical density (OD) at 450 nm was read using a microplate reader for evaluating the mice serum and mAb.

2.5. Evaluation of mAb with icELISA. A series of DAF standard in 0.01 M PBS was prepared (0, 0.3, 0.6, 1.2, 2.5, 5, 10, 20, and 30 ng/mL) for establishing the standard calibration curve to evaluate the LOD of the mAb. Each concentration was analyzed by optimized icELISA in triplicate. Nine FQ analogues including OFL, MOX, CIP, CIN, SAR, FLE, ENR, MAR, and PEF were used to evaluate the specificity of the mAb by the icELISA. Each measurement was analyzed in three replicates. Also, the concentrations of the nine FQ analogues were 0, 20, 60, 180, 540, 1080, 3240, and 9720 ng/mL. The cross-reactivity (CR) values were calculated with the following equation: CR (%) = (IC₅₀ value of DAF/IC₅₀ value of the analogue tested) × 100%.

2.6. Recovery Test in Meat. Standard calibration curve and recovery test in duck meat were performed as similar as 0.01 M PBS. Briefly, the duck from Jiangxi HuangshanghuanG Group Food Co., Ltd. was confirmed negative by Jiangxi Entry—Exit Inspection and Quarantine Bureau using LC/MS/MS. Five grams of ground duck samples was weighed into a polypropylene centrifuge tube (50 mL) for DAF detection. The duck samples were spiked with standard DAF at different amounts (10, 30, and 300 ng). The mixture was vortexed for 10 min using 5 mL of 0.2 M acetae buffer (pH = 5.6). After centrifugation at 8000g for 10 min, the supernatant (diluted to 10 mL with 0.01 M PBS) was used for the recovery test at the same icELISA conditions. Each spiked sample was repeated in quadruplicate. The OD at 450 nm was read. The recovery rate was the ratio of measured value to DAF amount spiked in duck meat. The measured value was calculated by the OD₅₀ value of the sample and the standard calibration curve for detecting DAN in meat. Also, the error bar was obtained by calculating the standard deviation (SD) of experimental data.

2.7. Preparation of Immunochromatographic Strips and Optimization of Key Parameters. GPNs were prepared in accordance with a previously reported method.22 The sample pad and NC membrane were pretreated in accordance with previous methods.23 Approximately, 0.74 μL/cm of DAF—OVA (0.6, 0.8, 1.0, 1.2, 1.4 μg/mL) was sprayed on the NC membrane as test (T) lines by using the BioDot XYZ platform. Goat antimouse IgG was sprayed on the NC membrane as control (C) line. The NC membrane was dried at 37 °C for 12 h. Finally, the sample pad, NC membrane, and absorbent pad were assembled as the immunochromatographic strip. Then, the GNP—mAb complex was prepared as follows. First, the pH (5, 6, 7, 8, and 9) of GNP solution was adjusted with 0.2 M K₂CO₃. Under gentle stirring, 100 μL of mAb (2, 2.5, 3.5, 4 μg/mL) diluted with ultrapure water was added dropwise to 1 mL of the GNP solution to form the GNP—mAb complex, which was added to the immunochromatographic strips as a signal tracer. Besides, the immunological kinetic curve between GNP—mAb and DAF—OVA on the T line was established by plotting the signal of the T line when the concentrations of DAF were 0 and 3 ng/g.
2.8. Analysis Performance of the mAb with the Immunochromatographic Strip. The PBS solution or supernantant of meat samples incubated with the GNP−mAb complex was added to the sample pad and immediately migrated toward the absorbent pad. During the migration process, DAF in the PBS solution or meat sample competitively bound to the GNP−mAb complex with the DAF−OVA on the T line, resulting in a regular color change of the T line. The result was detected with a portable GNP reader. To evaluate the analysis performance of the mAb with the immunochromatographic strip, a standard competitive inhibition curve was obtained by plotting $1 - T/T_0$ against the DAF concentrations.

3. RESULTS AND DISCUSSION

3.1. Principles of Preparing and Evaluating the mAb. The preparation of the mAb was based on hybridoma technology. Briefly, the process involves immunogen synthesis, immunization, cell fusion, positive cell screening, ascites collection, and purification. The evaluation of the mAb was based on icELISA and immunochromatographic strip. The principle of icELISA is that the analyte competes with the immobilized antigen to bind with the antibody. The method is particularly useful for detecting small molecule haptens with only one antigenic determinant, with a high sensitivity and high specificity. The process of preparation and evaluation is shown in Figure 1.

3.2. Characterization of the Immune and Coating Antigens. Antigens are important for the preparation of mAb. The immunogen and coating antigen were prepared by the active ester method as shown in Figure 2A. Also, they were characterized using a UV−vis spectrophotometer (Figure 2B). Figure 2B shows that the absorption peaks of DAF at 282 and 347 nm migrated to 285 and 328 nm after DAF conjugated with BSA, indicating that the DAF−BSA conjugate was successfully prepared. Similarly, the DAF−OVA conjugate was successfully prepared as confirmed by the appearance of two characteristic absorption peaks at 282 and 319 nm (Figure 2C).

3.3. Preparation and Evaluation of mAb with icELISA. The serum of mice immunized with DAF−BSA immunogen and positive clones was evaluated by icELISA. After repeating the cloning process three times, the cell line 1H1 and the corresponding antibody named mAb-1H1 with good specificity and sensitivity were obtained.
The affinity and inhibition of mAb-1H1 were evaluated with the developed icELISA. The calibration curve was constructed by plotting the OD_{450} values versus the logarithm of the DAF concentrations (0–30 ng/mL). As shown in Figure 3, the linear regression equation was \( y = -0.374 \ln(x) + 0.8092 \). The LOD was 0.2 ng/mL, which was defined as the mean of the negative sample minus threefold SD, and the IC_{50} was 1.39 ng/mL. The results indicated that the prepared mAb was more sensitive compared with polyclonal antibodies from rabbits. As shown in Table 1, mAb-1H1 showed high specificity except with CIP (1.93%) and OFL (1.88%). The same cyclopropyl and piperazine ring at the mother ring of CIP may be the reason for the low CR value. Given that their only difference is the heterocyclic ring (Table 1), the low CR value of OFL indicates that the recognition of the antigen and antibody was affected by the degree of hybridization.

3.4. Recovery Experiment of DAF in Meat. The standard calibration curve in meat was \( y = -0.455 \ln(x) + 1.8415 \) (Figure 4), and the LOD was 0.798 ng/g. Recovery experiments were conducted using the developed icELISA in duck samples. As shown in Table 2, the recovery rate ranged from 85.97 to 100.65%, and reproducibility (\( n = 4 \)) showed that the SD ranged from 0.003 to 0.025. Therefore, the developed icELISA was reliable and stable for the determination of DAF in duck meat.

3.5. Optimization of Key Parameters of the Immuno-chromatographic Strip. Some key parameters, including the pH of labeling the mAb, the concentrations of the mAb and DAF−OVA, and time, were optimized for the best performance of the immunochromatographic strip. pH was a decisive factor for forming GNP−mAb. The labeling efficiency gradually increased with the pH increasing from 5 to 6 and then dropped off quickly when the pH further increased to 9 (Figure 5A). Thus, pH = 6 was selected to form the GNP−mAb. The concentrations of the mAb and DAF−OVA directly affected the intensity of detection signals and inhibitory rate, which were the key factors determining the detection sensitivity of the immunochromatographic strip. Thus, the concentrations of DAF−OVA and the mAb were optimized as 1.2 mg/mL and 3.5 \( \mu \)g/mL, respectively, to ensure high signal value and inhibition rate (Figure 5B,C). Also, the immunological kinetic curve is shown in Figure 5D. The detection signal value gradually increased with time within 30 min and tended to stabilize after 30 min. Thirty minutes was selected as the final quantitative detection of DAF.

3.6. Analysis Performance of mAb with the Immuno-chromatographic Strip. PBS solution or meat samples spiked with different concentrations of DAF were analyzed using the

### Table 1. CR of mAb-1H1

| Drugs | Structure | IC_{50} (ng/mL) | CR (%) |
|-------|-----------|-----------------|--------|
| DAF   |           | 0.39            | 100    |
| CIP   | ![Chemical Structure](https://example.com/structure_cip.png) | 20.2   | 1.93   |
| OFL   | ![Chemical Structure](https://example.com/structure_ofl.png) | 20.8   | 1.88   |
| MOX   | ![Chemical Structure](https://example.com/structure_mox.png) | >200   | <0.2   |
| ENR   | ![Chemical Structure](https://example.com/structure enr.png) | >200   | <0.2   |
| PEF   | ![Chemical Structure](https://example.com/structure pef.png) | >200   | <0.2   |
| SAR   | ![Chemical Structure](https://example.com/structure sar.png) | >200   | <0.2   |
| LOM   | ![Chemical Structure](https://example.com/structure lom.png) | >200   | <0.2   |
| MAR   | ![Chemical Structure](https://example.com/structure mar.png) | >200   | <0.2   |
| FLE   | ![Chemical Structure](https://example.com/structure fle.png) | >200   | <0.2   |
| CIN   | ![Chemical Structure](https://example.com/structure cin.png) | >200   | <0.2   |

### Table 2. Recovery Experiment of DAF Using the Developed icELISA in Duck

| spiked DAF (ng/mL) | mean (ng/mL) | SD  | recovery (%) |
|--------------------|--------------|-----|--------------|
| 10                 | 8.60         | 0.025 | 85.97            |
| 30                 | 28.94        | 0.025 | 96.47            |
| 300                | 301.95       | 0.003 | 100.65           |

Figure 4. Calibration curve for the detection of DAF in duck meat based on icELISA.
lateral-flow immunochromatographic strip. The standard curve for detecting DAF in PBS was 
\[ y = 0.3149 \ln(x) + 0.0533 \] 
\( R^2 = 0.9881 \), and the LOD was 0.24 ng/mL (Figure 6A). Figure 6B shows that the cut-off was 3 ng/mL for DAF in PBS. As shown in Figure 7A, the standard competitive inhibition curve in meat was 
\[ y = 0.1864x - 0.02 \] 
and the LOD was be 0.95 ng/g. Figure 7B shows the sensitivity of the test strips, and the cut-off value was 5 ng/g, which indicated that the lateral-flow immunochromatographic strip developed here is sensitive, accurate, and suitable for DAF detection in meat samples.

Besides, the method we developed have been compared with other detection methods in detection range and detection limit.
and can be considered effective screening methods for foodstuffs. Overall, the developed icELISA and immunochromatographic strip were reliable for the rapid detection of DAF in meat and can be considered effective screening methods for food safety and quality management.

4. CONCLUSIONS

In this study, a sensitive and specific mAb against DAF was prepared for the first time. An icELISA based on the mAb was developed for DAF detection in duck meat. The IC_{50} of ELISA was 1.39 ng/mL. The CR experiment indicated that the developed icELISA was highly specific and had low CR with other FQ drugs. An immunochromatographic strip based on the mAb was also developed, with a cut-off value of 5 ng/mL in duck meat. Overall, the developed icELISA and immunochromatographic strip were reliable for the rapid detection of DAF in meat and can be considered effective screening methods for food safety and quality management.

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Notes

Compliance with ethical standards.

The authors declare no competing financial interest.

The authors declare that no competing financial interest was declared.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Ethical approval: This article does not contain any studies with human participants performed by any of the authors.

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