Production and Characterization of Monoclonal Antibodies against Norfloxacin

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Abstract

To monitor the abuse of Norfloxacin (NFLX), developing a monoclonal antibody (mAb) based enzyme linked immunosorbent assay (ELISA) kit is our future goal. For this purpose, Hybridomas were prepared by fusing NS0 mouse myeloma cells with splenocytes isolated from immunized BALB/c mouse. Noncompetitive and competitive indirect ELISA was employed to screen positive cell clones. Caprylic acid ammonium sulphate (CAAP) method was used to purify NFLX mAb and the Batty saturation method was used to determine the affinity constant (Ka). Finally, six hybridoma cell lines named N1-A2, N2-B3, N2-B5, N3-C6, N4-D5, and N4-D8 were screened out, their corresponding mAbs were of the IgG 1 isotype with k light chain, and the Kas of all mAbs were between 3.2×10⁹ and 2.6×10¹⁰ L/mol. Based on the square matrix titration, two representative icELISA curves were established. The dynamic range for N3-C6 in assay buffer was from 0.003 to 38 ng/mL, with LOD and IC₅₀ value of 0.002 ng/mL and 0.16 ng/mL, respectively. As to N2-B5, it was from 0.004 to 26 ng/mL, with LOD and IC₅₀ value of 0.002 and 0.22 ng/mL, respectively. Except for a high cross-reactivity (CR) to Pefloxacin (33.6%) and Lomefloxacin (21.8%), this mAb originated from N3-C6 exhibited negligible CR values to other chemicals. Therefore, the established hybridomas put a potential for the development of a rapid test kit and provide an alternative method for the detection of NFLX residues in food producing animals.

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Keywords: Norfloxacin; Monoclonal antibody; Indirect competitive ELISA; Characterization

1. Introduction

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Quinolones are an important group of synthetic antibiotics with antibacterial action. These compounds have a carboxylic acid group in position 4, and are frequently referred to as 4-quinolones [1]. The original quinolones have only modest activity against Enterobacteriaceae and some other facultative Gram-negative bacteria. However, a new class of antibiotics called fluoroquinolones (FQs) started in the early 1980s with the discovery that a combination of a fluorine atom at position 6 and a piperazinyl group at position 7, which resulted in a broad and potent antimicrobial activity [2]. This combination produced a broad spectrum of activity and better pharmacokinetic profile against Gram-negatives and some Gram-positives bacteria with antibacterial activities 1000 times more potent than those observed in the quinolones. The FQs improved the treatment of infectious diseases due to their fewer toxic side effects and also enhanced pharmacokinetics properties, including resistant strains. This class of antibiotics is the first-line therapy for complicated urinary tract and bacterial diarrhea. They are also alternative agents for the treatment of many sexually transmitted diseases, as well as osteomyelitis, some cases of wound infection and selected respiratory infections [3].

In recent years, there have been increasing concerns about the use of such compounds in animal industry for the control of early mortality, because it may contribute to the acquisition of resistance by bacteria, which would in turn, lead to a reduction in the efficacy of such compounds for treating infections in humans. Furthermore, following the administration of drugs to animals, residues or metabolites may persist in foods, thereby raising potential human health concerns [4]. Therefore, in order to ensure that consumers are not exposed to FQs residues at potentially harmful concentrations, the use of antibiotics in animal production is strictly regulated and maximum residue levels (MRLs) have been established by many countries. The European Union MRLs have been set for Enrofloxacin (+ Ciprofloxacin) (100 μg/kg), Flumequine (50 μg/kg), Marbofloxacin (75 μg/kg), and Danofloxacin (30 μg/kg) (03/2004) by Council Regulation EEC/2377/90 (and amendments) [5].

Traditionally, FQs residue analysis has relied upon classical methods, such as high performance liquid chromatography (HPLC) [6], liquid chromatography-mass spectrometry (LC-MS) [7], and LC-MS/MS [8], and other quantitative methods. Chromatographic techniques generally require highly skilled personnel, laborious sample pretreatment and high-cost complex equipment. In this study, our main goal was to produce high-sensitivity monoclonal antibodies displaying excellent affinity and specificity towards Norfloxacin (NFLX), and optimize the ELISA protocols based on selected monoclonal antibodies. This work potentially optimizes the pre-treatment procedures for LC-MS and GC-MS detection, lay a solid foundation for NFLX-kit and test strip development.

2. Materials and Methods

2.1. Materials and equipments

Norfloxacin, Enrofloxacin, Ofloxacin, Pefloxacin, Lomefloxacin and Danofloxacin were provided by Sigma (St. Louis, MO). NFLX–BSA as immunogen and NFLX-OVA as coating antigen were conjugated in our laboratory. FCA and FIA were obtained from Pierce. GaMlgG-HRP (whole molecule specific) was purchased from Sino-American Biotechnology Company (Shanghai, China). HAT and HT were obtained from Sigma-Aldrich (USA). RPMI-1640 with L-glutamine was obtained from Gibco. Polyethylene glycol 1500 (PEG 1500, 50%) was from Roche Diagnostics Corporation (Indianapolis, USA). A mouse monoclonal antibody isotyping kit was purchased from Pierce Biotechnology, Inc. (Rockford, II, USA). TMB, phenacetin, urea peroxide were obtained from Sigma Company. All other solvents and reagents were of analytical grade or higher, unless otherwise stated.

A spectrophotometric microtitre reader (MULTISKAN MK3, Thermo Company, USA) was used for absorbance measurements. A GS15R high speed refrigerated centrifuge were supplied by Thermo
Company (USA). CO₂ incubator from RS-Biotech (Galaxy S+, UK) was used for cell cultivation. SW-CJ-2FD Superclean Bench was purchased from Suzhou purification equipment Co., Ltd (Suzhou, China). Inverted microscope (TS100-F, Nikon Company, Japan) was used for cell observation.

2.2. Mouse immunization

NFLX-BSA (100 μg in 0.25 mL of phosphate-buffered saline (PBS), mixed with an equal volume of FCA to form an emulsion) was injected intraperitoneally into five female BALB/c mice. For subsequent boosters, FIA was substituted for the FCA as an emulsifier, and mice were injected 100 μg of immunogen in booster injections every three weeks. After the third booster immunization, blood was obtained from the coccygeal vein section, and the sera were checked for their titer and ability to compete with NFLX. The mouse whose antiserum showed strongest competition toward NFLX was selected for the fusion experiment. Four days prior to splenocyte harvest, the mouse was injected with 100 μg of immunogen divided equally in intravenous and intraperitoneal injections.

2.3. Fusion of myeloma and spleen cells

The standard procedure for monoclonal antibody generation was described previously by Chen et al [9]. Briefly, NS0 myeloma cells were passed through medium containing 8-azaguanine and then grown for 4-5 days at 37 °C in 5% CO₂ atmosphere. On the day of fusion, the mouse (that received the booster injection) was sacrificed by cervical dislocation and the spleen was removed aseptically. The splenocytes were isolated and fused with myeloma cells at a 10:1 ratio using PEG 1500 as the fusing agent, followed by gentle stirring for 1 min. The resulting mixture was kept still at 37 °C for 1.5 min, and then 40 mL of HAT-1640 medium (supplemented with 15% FBS) was slowly added to the fused cells. The fused cells were then distributed into 96-well culture plates, in which mouse peritoneal macrophages were prepared on the day before the fusion and were grown with the selective HAT medium.

2.4. Hybridoma production, selection, and cloning

10-14 days after fusion, supernatants of hybridoma colonies were recovered and screened using a combination of noncompetitive and competitive indirect ELISA. Well cultures showing significant NFLX recognition activity were expanded from the culture in the 96-well plate to a 24-well plate, and subcloned three times by limiting dilution. The wells picked for expansion were viewed under a microscope to confirm the presence of a single cell source, ensuring their monoclonal origin, and the HT medium was gradually replaced by complete medium. After hybridomas became dense in the 24-well plate, they were transferred to 50 or 100 mL culture flasks. Hybridomas cells were collected, centrifuged and the supernatants were stored at -20 °C until used. Colonies of interest were frozen in culture medium containing 10% dimethyl sulphoxide (DMSO) and cryopreserved in liquid nitrogen, followed by defrosting three times to select the stable antibody producing clones.

A mature female BALB/c mouse was injected intraperitoneally (i.p.) with 0.5 mL of paraffin 10 days before receiving an i.p. injection of the positive hybridoma cells suspended in RPMI 1640 medium. Ascites fluid was collected 10 days after the injection and then stored at -20 °C until use. Purification of mAb was performed according to the modified caprylic acid ammonium sulphate precipitation (CAASP) method described before [10].
2.5. Characterization of mAbs

The class and subclass of the isotypes of the purified antibody were determined by using a mouse monoclonal antibody isotyping kit. Measurement of monoclonal antibody affinity \((K_a)\) was carried out according to the procedure described by Wang et al. [11]. An indirect competitive ELISA (icELISA) standard curve was developed based on the mAbs. Sensitivity was calculated using the IC\(_{50}\) values, which represented the concentration ofNFLX that produced 50% inhibition. The detection of limit (LOD) was defined as the lowest concentration that exhibits a signal of 15% inhibition [12]. The dynamic range was calculated as the concentration of the analyte providing a 20–80% inhibition rate (IC\(_{20}\)–IC\(_{80}\) values) of the maximum signal. Specificity was expressed by the cross-reactivity, which was calculated as: (IC\(_{50}\) of NFLX)/ (IC\(_{50}\) of competitors) ×100. The lower the CR is, the higher the specificity of NFLX mAb is.

3. Results and Discussions

3.1. Selection of potential cell-fused mice

Five mice were immunized with the NFLX-BSA conjugate following the standard protocols described above, while NFLX-OVA were coated onto ELISA plates to determine the titer and inhibition level of antisera. After three subsequent injections, four of the five mice immunized with NFLX-BSA produced antisera with significant anti-NFLX activities. From the obtained inhibition curves, NO.4 mouse afforded the most sensitive IC\(_{50}\) value (1.75 ng/mL), that was selected for further use.

3.2. Production and characterization of mAbs

Ten days following the fusion, growing hybridoma cell clones could be observed in many wells of the seeded 96-well plates. The fusion rate of the mouse spleen cells with myeloma cells was about 81%. Supernatants of all wells were screened by simultaneous noncompetitive and competitive assays, and the positive well rate was 26%. Selection of clones from these positive cultures by limiting dilution led to six stable hybridoma cell lines. These monoclonal cultures and their corresponding cell lines were named N1-A2, N2-B3, N2-B5, N3-C6, N4-D5, and N4-D8. Using a mouse monoclonal antibody isotyping kit, all six antibodies were of the IgG\(_1\) isotype with \(k\) light chain. And the protein concentrations of all mAbs were between 1.6–2.8 g/mL. Finally, these six hybridomas were expanded and stored in liquid nitrogen. The mAbs from culture supernatants and ascite liquids were purified and characterized. Based on the results of the checkerboard titration, the antibody titers and IC\(_{50}\) values were determined. From the obtained results, the most sensitive hybridoma named N3-C6 showed the IC\(_{50}\) value of 0.16 ng/mL.

The affinity of an antibody for its corresponding antigen is crucial parameters affecting the performance of an immunoassay, and high-affinity antibodies can produce sensitive IC\(_{50}\) values. In our study, the affinity constant \((K_a)\) of six selected hybridomas were between \(3.2\times10^{9}–2.6\times10^{10}\) L/mol (Fig. 1), in which N2-B5 and N3-C6 were used for subsequent immunoassay development.
3.3. Hybridoma stability experiments

Six hybridoma cell lines producing the interested antibodies were identified by stability verification. Through three times of frozen and defrosted procedure, the performances of N2-B5 and N3-C6 were more consistent in the experiments. The results are shown in Fig. 2.

3.4. Establishment of the icELISA standard curves

To ascertain the applicability of N2-B5 and N3-C6 monoclonal antibodies generated in this study, icELISA standard curves were investigated. It is well known that working concentrations of antibody and coating antigen are crucial factors for the sensitivity of ELISA methods. For this reason, checkerboard titrations were performed, taking into account the optimal dilutions. The optimal reagent concentrations were determined when the maximum absorbance ($A_{\text{max}}$) was around 1.0, and the dose-response curve of inhibition ratio versus the NFLX concentration pursued the lowest $IC_{50}$ values. As can be seen, the optimum concentration of coating antigen was 0.3 $\mu$g/mL and mAb was 1:10,000 dilutions. Based on the checkerboard titrations, two competitive curves were obtained with the icELISA format (Fig. 3). For the clone of N3-C6, this assay allowed the detection of NFLX (20-80% inhibition of color development) from 0.003 to 38 ng/mL, with an $IC_{50}$ value of 0.16 ng/mL, and LOD value of 0.002 ng/mL. As to clone of N2-B5, the icELISA allowed the detection of NFLX from 0.004 to 26 ng/mL, with an $IC_{50}$ value of...
0.22 ng/mL, and LOD value of 0.002 ng/mL.

Fig. 3. Optimized icELISA inhibition curves based on N3-C6 and N2-B5

3.5. Specificity

Specificity was evaluated by determination of the cross-reactivity values. 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 cross-reactivity rates for each compound are presented. Except for a high cross-reactivity to Pefloxacine (33.6%) and Lomefloxacin (21.8%), this assay exhibited negligible CR values to other chemicals. It proves that it is highly specific for the NFLX mAbs.

Acknowledgements

This work was supported by Henan Innovation Project for University Prominent Research Talents (2010HASTIT026).

References


