

Research Article

Preparation and Enzyme-Linked Immunosorbent Assay of Monoclonal Antibody Against Ampicillin

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Abstract: Ampicillin (AMP) was coupled with mκKLH by the method of EDC intermediate and injected into Balb/c mice as immunogen. The hybridomas were obtained by fusing mouse myeloma cells SP2/0 with splenocytes from the mice immunized with AMP-KLH. The chromosomal numbers were 97-104, the results indicated that the subclass of the McAb was IgG2a, the titer of ascitic fluid was 2×10^6 , the affinity constant was 3×10^{-10} mol/L, the molecular weight was 154KD. The cross-reactivities of McAb to other antibiotics were below 0.01%. The linear detecting range of calibration curves to detect AMP was 0.5 ng/mL-100 ng/mL. The formular was $Y = 0.0569X + 0.1308$, $R^2 = 0.9948$. The limit of detection was 0.3 ng/mL.

Keywords: Ampicillin, ELISA, monoclonal antibody

INTRODUCTION

Penicillin is one of the oldest antimicrobial agents widely used for controlling of cow mastitis and treatment of urinary tract, reproductive tract, gastrointestinal tract and respiratory infections. Due to specific side effects related to allergic reaction and drug resistance, the use of penicillin to animals and the penicillin residues in animal-based food are strictly controlled and monitored in many countries. A variety of analytical methods have been developed for penicillin residue assays, including the initial microbial growth inhibition test and the relative sensitive instrumental analysis method (Lih *et al.*, 1996; Sorensen *et al.*, 1997; Fontes *et al.*, 1996). At present, the high-performance liquid chromatography (HPLC), immunoassay and microbioassay are frequently used for penicillin residue assays. Of these, the HPLC is sensitive and highly efficient, thus is commonly used at a global scale. However, this method requires special detection equipment. By comparison, microbioassay is easy to operate but its sensitivity is relatively low. Immunoassay is a simple, rapid, sensitive analytical method with broad application prospects, which combines the antigen-antibody reaction with sensitive detection system (Li *et al.*, 2002). In addition, several new detection methods have been developed in recent years, such as capillary electrophoresis (Ahrer *et al.*,

2001) and penicillin receptor protein test (Setford *et al.*, 1999). However, these methods have not been widely used despite they meet the requirement in terms of maximum residue limits (MRL). In the present study, hybridoma technology was employed to establish hybridoma cell lines capable of producing monoclonal Antibody Against Ampicillin (AMP) and a rapid AMP detection technology was developed via Enzyme-Linked Immunosorbent Assay (ELISA).

MATERIALS AND METHODS

The AMP standard, Bovine Serum Albumin (BSA), Freund's adjuvant, O-Phenylenediamine (OPD), PEG solution, horseradish peroxidase (HRP)-labeled goat anti-mouse IgG, HAT and HT solutions and the IgG subclass reagents were purchased from Sigma; low-glucose dry-powdered DMEM and high-quality fetal bovine serum were purchased from Hyclone; EDC coupling kit was purchased from Pierce; Balb/c mice were purchased from the Laboratory Animal Center of the Chinese Academy of Military Medical Sciences; and SP2/0 myeloma cells were stored in the High-tech Laboratory of the Institute of Animal Husbandry and Veterinary Medicine, Beijing Academy of Agriculture and Forestry Sciences.

Methods:

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Synthesis and animal immunity assay of AMP-holoantigen: Synthesis of AMP-BSA and AMP-KLH: The AMP was coupled with BSA and KLH using the EDC method. The obtained AMP-KLH was used as the immunogen and AMP-BSA as the coating antigen. Intraperitoneal injection was used for animal immunity assay and cell fusion was performed on the spleen of selected mice with high serum titer.

Preparation and screening of hybridoma cells: Myeloma cells (1×10^7 cells) and immune cells from mouse spleen (1×10^8 cells) were harvested at the logarithmic phase and fused with 50% PEG according to the conventional method. The supernatant was cultured by indirect ELISA and strong positive clones were chosen. The cells were cloned for 3 times by limited dilution and the hybridomas with a positive rate of 100% were further cultured and frozen in liquid nitrogen.

Characterization of anti-AMP-McAb: The McAb supernatant (or McAb ascites) of cell culture was consecutively diluted from 1000-fold using the doubling dilution method. To the diluted McAb supernatant was added the microtiter plates coated with AMP-BSA, followed by HRP-conjugated goat anti-mouse IgG. Two wells were established for the positive control (positive mouse serum), negative control (non-immune mouse serum) and Blank Control (PBS). The test result was considered to be positive when the OD value at 490 nm was greater than or twice that of the negative control. The titer was taken as the highest dilution of the positive wells.

The hybridomas in the logarithmic phase were added with 0.1 $\mu\text{g}/\text{mL}$ colchicine and then cultured for 6-10 h at 37°C. Then, the cells were collected, centrifuged at 200g for 10min, treated with 5 mL of 0.075 mol/L KCl at 37 °C and fixed with acetic acid and methanol (1:3, v/v) on a glass slide for three times. The slide was air-dried, stained with 10% Giemsa for 10 min, washed with distilled water and then naturally dried. One-hundred complete intermediate nucleated cells were examined by light microscopy and the number of the chromosomes was recorded.

Cross-reaction with KLH and BSA: The cells on microtiter plates coated with AMP-BSA, KLH or BSA were sealed with PBS containing 5% skim milk powder and then added with diluted monoclonal antibody. After the reaction, HRP-conjugated goat anti-mouse IgG was added. The color reaction was achieved using an OPD chromogenic substrate system and the OD value of microtiter plates was determined at 490 nm.

Molecular weight determination. Purified ascites were diluted to an appropriate range and electrophoresized with polyacrylamide gel and 10%

separating gel. After electrophoresis, the gels were stained and bleached for observation.

Cross-reaction with β -lactams and other common antibiotics: The working solutions of tested drugs were prepared at the concentration of 100 μg , 10 μg , 1 μg and 0.1 $\mu\text{g}/\text{mL}$. The concentration-inhibition rate curve was established and used to calculate the cross-reaction rate: $\text{Cross-reaction rate} = (\text{AMP IC}_{50}/\text{Test drug IC}_{50}) \times 100\%$ (IC_{50} is the inhibition rate of 50%)

Identification of immunoglobulin class and subclass: The immunoglobulin class and subclass were identified using the Sigma antibody subclass identification kit following the manufacture's instruction.

Determination of affinity constants: The antigen-coated microtiter plates diluted to 2, 1 and 0.5 $\mu\text{g}/\text{mL}$ were transferred to separate wells (100 μL each) for incubation (overnight, 4 °C). After sealed with 1% BSA at 37 °C for 1 h, the microtiter plates was added with serial dilution of monoclonal antibodies with known concentrations and incubated at 37 °C for 1 h. Thereafter, the enzyme-labeled secondary antibody was added at an appropriate concentration and the OPD substrate buffer was used for color reaction. The reaction was terminated by 2 mol/L H_2SO_4 and the OD value of each well was measured at 490 nm. Three measurement curves were made using the concentrations of monoclonal antibody as the abscissa and the corresponding OD values as the ordinate. The concentration of monoclonal antibody corresponding to the OD value of 50%, i.e., $[\text{Ab}]_t$, was obtained based on that corresponding to the OD of 100% (the plain part near the top of each line). Accordingly, the 3 values $[\text{Ab}]_t$, $[\text{Ab}']_t$ and $[\text{Ab}'']_t$ were obtained and the K value was calculated as follows: $K_1 = 1/2(2[\text{Ab}']_t - [\text{Ab}]_t)$, $K_2 = 1/2([\text{Ab}'']_t - [\text{Ab}']_t)$ and $K_3 = 3/2([\text{Ab}'']_t - [\text{Ab}]_t)$. The average value of K_1 , K_2 and K_3 were taken as the affinity constant.

Preparation of standard curve: The standard curve was prepared using the indirect competitive ELISA method. The standard was doubly diluted to 0.01–100 ng/ml. The obtained OD value without AMP inhibition was B0 and that with AMP inhibition was B. The standard curve was prepared using the inhibition rate $[(B_0 - B)/B_0]$ as the coordinate and the negative logarithm of the AMP concentration as the abscissa. The regression equation was established for the standard curve.

Detection of AMP in commercial pure milk: The AMP was added to the commercially pure fresh milk to a certain concentration (0-100 ng/mL), oscillated at 4°C overnight and centrifuged at 5000 rpm for 10 min. The supernatant was collected and transferred to the

microtiter plate (100 μ L per well). The standard curve was prepared using the indirect competitive ELISA method and the detection limit of AMP in milk was calculated and.

RESULTS AND DISCUSSION

The fusion rate of cell colony was 99% three days after fusion. Subsequent subcloning for 3 times screened 8 hybridomas with high specificity and high affinity. These hybridoma stably secreted antibody after several passages, cryopreservation and recovery.

The titers of ascites were up to 2×10^6 , significantly higher than that of the cell supernatant (1:6400). The concentration of purified monoclonal antibody of ascites was 2 mg/mL and that of the cell supernatant was 1.5 mg/mL after salting out and crude extraction with saturated ammonium sulfate. The titer of purified cell supernatant was 1:102400. Of the 8 hybridomas, 3D12 was relatively stable in terms of secretion of antibodies and titer. Therefore, the 3D12 hybridoma lines were selected for subsequent experiment.

The chromosome numbers of known mouse spleen cells and SP2/0 myeloma cells were 40 and 60-70, respectively, whereas that of hybridoma obtained in this study was 97-104 (Fig. 1).

The molecular weight of the purified monoclonal antibody was measured by SDS-PAGE electrophoresis. According to the linear relationship between the molecular weight and the migration rate, the molecular weights of the light chain and the heavy chain of monoclonal antibody were 26 kd and 51 kd, respectively and the molecular weight of the monoclonal antibody was 154 kd (Fig. 2). The subtype of 3D12 strain was IgG2a.

Cross-reaction of McAb to other drugs (Table 1). The protein concentration of the monoclonal antibody was 1.5 mg/mL. According to the formula $K=3 \times 10^{10}$ mol/L, the concentrations of its dilutions were 1×10^{-3} , 5×10^{-4} , 2.5×10^{-4} , 1×10^{-4} , 5×10^{-5} , 2.5×10^{-5} , 1×10^{-5} and 5×10^{-6} mg/mL, respectively (Fig. 3).

The regression equation showed a good linear relationship between the concentration range of 0.5-100 ng/mL. The linear regression equation between inhibition rate and concentration ($-\log C$, 1 mg/mL mother liquor) was $Y = 0.0569 X + 0.1308$ and the correlation coefficient was $R^2 = 0.9948$. Based on the concentration corresponding to 10% inhibition rate, the detection limit of AMP was calculated to be 0.3 ng/mL. As for AMP added to the commercially-available, pasteurized pure milk, the minimum detection limit was 0.4 ng/mL (Fig. 4).

Immunoassay, as an analytical technique developed in recent years, has been widely used in residue analysis of veterinary drugs, pesticides and other small molecule compounds. Antibody is the core reagent of immunoassay technology and its quality greatly affects the standardization of the detection methods (Wang and Li, 2000). Although the polyclonal

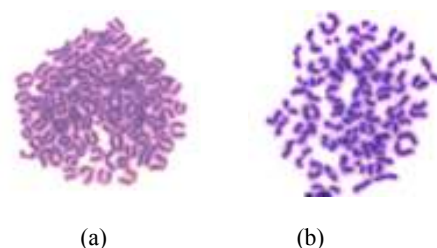


Fig. 1: The number of hybridism cell chromosome; (a) Chromosome of hybridism cell; (b) Chromosome of SP2/0

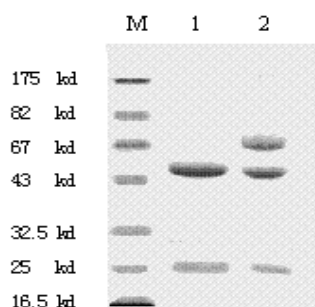


Fig. 2: The SDS-PAGE of monoclonal antibody against ampicillin

1 is the octanoic acid-ammonium sulfate purification method of antibody; 2 is unpurified mice ascites; M is Mark of molecular weight

Table 1: The cross reaction rate of monoclonal antibodies to antibiotics

Antibacterial drugs	Cross reaction rate (%)
Ampicillin	100
Ampicillin sodium	36
Penicillin sodium	< 0.007
Cefradine	< 0.001
Ceftiofur	< 0.001
Kanamycin	< 0.001
Streptomycin	< 0.001
Sulfadiazine	< 0.001
Gentamicin	< 0.001

antibody prepared by the application of small molecule haptens and carrier protein conjugate as the immunized animal contains an antibody against small molecules, it has two disadvantages:

- The low binding specificity of the polyclonal antibodies produced by the conjugates and small molecule antigen often leads to strong cross reaction and causes false-positive results of immunoassay.
- The carrier protein has many B cell epitopes, which stimulate the body to produce a large number of antibodies against the carrier protein, thus reducing the number of antibodies against small molecules and the affinity. By comparison, the monoclonal antibody is advantageous in terms of high specificity and easy control and standardization. Therefore, it has broader application prospects than

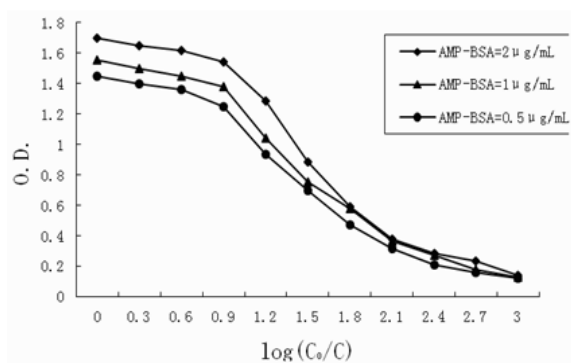


Fig. 3: The affinity constant of monoclonal antibody against Ampicillin

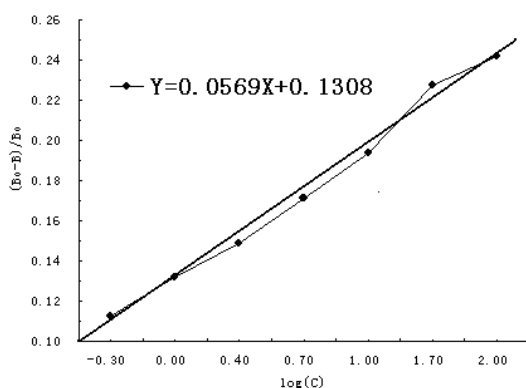


Fig. 4: The standard curve of monoclonal antibody against Ampicillin

polyclonal antibody in immune analysis of drug residues (Tan and Shi, 2000).

In the ELISA reaction system, multiple factors can affect the preparation of standard curve, including pH value, ionic strength, organic solvent, reaction temperature, reaction time and the amount of reactants and sample pretreatment method. The hydrogen bonding between antigen and antibody as well as ionized antibody ligand are substantially affected by pH values, which in turn affects the coating antigen and the molecular structure of the sample. Within a certain range of ionic strength, the high ionic strength can reduce the adsorption of the non-specific antibody. This is because the charge involved in the non-specific reactions can be covered by the large number of ions. However, if the ion concentration is too high, the specific antigen-antibody reaction will be affected and the detection sensitivity will decline greatly. The organic solvent may destroy the van der Waals force and the hydrophobic interaction between antigen and antibody, thus dissociating the antigen-antibody complex. In real practices, the pH value, ionic strength and organic solvent in the reaction system are affected by screening of various working fluids. In the present

study, the working fluids were screened to ensure the compliance of the antigen-antibody reaction.

The monoclonal antibody against AMP prepared in the present study was characterized with high titration, specificity and stability. The detection limit of the standard curve prepared for AMP detection was lower than the national maximum residue limit of ampicillin in animal food as required. Further test of the ELISA kit and test strip for AMP residue detection using this antibody is ongoing.

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