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Immunochromatographic strip development for ultrasensitive analysis of aflatoxin M1

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A specific monoclonal antibody against aflatoxin (AF) M1 was prepared by a duo-immunogen immunization strategy. After two immunizations of Balb/c mice with AFB1 keyhole limpet hemocyanin (KLH) conjugates and followed by five subsequent immunizations of AFM–KLH, the mice secreting antibody against AFM1 were selected for cell fusion and a 7C6-H1 cell line was obtained to produce the antibody. The 50% inhibition rate of the prepared antibody was calculated as 0.034 ± 0.002 μg L^{-1}. The anti-AFM1 antibody was characterized with high affinity to AFM1 (1.28×10^9 mol L^{-1}) and low cross-reactivity to related AFs (<5%). With a competitive format, an immunochromatographic strip was developed using AFB1–bovine serum albumin as the immobilized antigen and anti-AFM1 antibody labeled with gold nanoparticles as tracers. Without any sample preparation, the strip could be directly applied to detect AFM1 contamination in liquid milk. The minimum cut-off concentrations were 0.2 μg L^{-1} and 1.6 μg kg^{-1} in liquid milk and powdered milk, respectively, by assessment with the naked eye. Detection of real samples and spiked milk samples indicated the potential of this strip in routine AFM1 monitoring.

1 Introduction

Aflatoxin (AF) M1 is regarded as a transformed compound due to enzymatic hydroxylation of AFB1.[1,2] This conversion is found when dairy cows digest feed polluted by AFB1. Many studies have confirmed AFM1 hepatotoxicity and carcinogenicity in humans.[3] Hence, AFM1 is regarded as a group I carcinogen by the International Agency for Research on Cancer (IARC).[4] The risk of AFM1 in milk is a major concern due to the high daily consumption rates of milk worldwide.[5–7]

The tolerance level for AFM1 in milk products has been set at 0.5 μg L^{-1} (Chinese National Standard GB2761). Immunoassay, is a sensitive, low-cost and fast detection technique that has been reported to detect AFM1. Magliulo *et al.*[8] established a chemiluminescent enzyme immunoassay with a limit of quantification of 1 ppt in milk analyses. Pei *et al.*[9] developed a monoclonal antibody against AFM1 to analyze milk samples with limit of detection 0.04 ng mL^{-1}. Based on a polyclonal antibody, Thirumala-Devi *et al.*[10] developed an enzyme-linked immunosorbent assay (ELISA) to detect AFM1. Extraction with methanol and by dilutions, Thirumala-Devi *et al.* invetigated AFM1 contamination in milk products in India, and 146 of 280 samples contained up to 48 μg L^{-1} AFM1. Similarly, Anfossi *et al.*[11] established an ELISA to measure AFM1 in dairy products. A limit of detection (LOD) of 5 μg kg^{-1} was obtained after pre-preparation steps. Using nanomaterials as probes, some sensitive immune sensors have recently been developed to detect AFM1 in milk products.[12–14] The lateral chromatography strip is very useful during field analysis. Wang *et al.*[15] reported an immunochromatographic strip based on polyclonal antibodies in 2011. The cut-off concentration of AFM1 was 2 μg L^{-1}. Salter[16] reported a test strip based on Charm Rapid One Step Assay with a positive/negative interpretation value of 0.5 μg L^{-1}. Anfossi[17] also developed an immunochromatographic strip to detect AFM1 in milk with a polyclonal antibody. Using a color reader, the LOD was calculated as 20 ng L^{-1} for pretreated milk samples.

Direct detection is a major challenge in trace analysis. In the present study, we developed an immunochromatographic strip to detect AFM1 specifically in liquid milk directly with a cut-off concentration of 0.2 μg L^{-1} (naked eye observation). The whole detection cycle could be completed within 15 min.

2 Materials and methods

2.1 Reagents and materials

AFM1, B1, G1 and G2 and other related mycotoxins including ochratoxin A (OTA), zearalenol (ZEN) and deoxynivalenol (DON) were purchased from Sigma (St. Louis, MO, USA). Goat anti-mouse IgG, bovine serum albumin (BSA), ovalbumin (OVA), and hemocyanin from *Megathura crenulata* (KLH) were purchased from Solarbio Science & Technology Co. Ltd. (Beijing, China).

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Poly(ethylene glycol) (PEG, Hybri-Max, mol wt 3000–3700), *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC·HCl), *N*-hydroxysuccinimide (NHS), and carboxymethoxylamine hemihydrochloride (CMO) were obtained from J&K Chemical Co. (Shanghai, China). RPMI 1640 cell culture medium and fetal calf serum were bought from HyClone (Sunshine Biotehnology Co. Ltd, Nanjing, China). The Sp2/0-Ag14 murine myeloma cell line was obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China). QuickAntibody adjuvant was supplied by Kang Biquan Biotechnology Co. (Beijing, China). The IsoQuick Kit for Mouse Monoclonal Isotyping was purchased from Youlong-Bio Co. (Shanghai, China).

Nitrocellulose (NC) membranes were purchased from Millipore Corporation (Bedford, MA, USA). Nitrocellulose high-flow plus membranes (Pura-bind RP) were purchased from Whatman-Xinhua Filter Paper Co. Ltd. (Hangzhou, China). Glass fiber membranes (CB-SB08) used for sample padding, the polyvinylchloride (PVC) backing material, and absorbance pads (SX18) were supplied by Goldbio Tech Co. Ltd. (Shanghai, China). All other chemicals and reagents (analytical grade or higher) were purchased from East China Chemicals Co. Ltd. (Shanghai, China).

2.2 Instruments

The Dispensing Platform (BioJet Quanti3000 dispenser) and the CM4000 Guillotine Cutting Module (BioDot Inc., Irvine, CA, USA) were used to prepare the test strips. The BioDot TSR3000 Membrane Strip Reader was used to identify the depth of color of the test line. The Fresco 17/21 centrifuge was purchased from Thermo Scientific (Heraeus, Germany). The UV-Vis spectrophotometer was purchased from Unico Analytical Instrument Company (Shanghai, China). The pH was adjusted with an acidometer (Tianda Apparatus Co. Ltd., Shanghai, China).

2.3 Preparation of monoclonal antibody against AFM1

Two haptens were prepared by inserting a carboxyl to the cyclopentenone of AFM1 and AFB1 as described by Chu[18] and Wang.[15] The modified AFM1 or AFB1 was conjugated with carrier protein (OVA, BSA and KLH) using an activated ester method. The KLH conjugates of AFM1 and AFB1 were injected into mice as immunogenic antigens. Six-week-old female Balb/c mice were immunized at a 3 week interval. For the first and second immunization, 25 μg (50 μL) AFB1–KLH was mixed with adjuvant (50 μL) and injected intramuscularly. The subsequent immunization was carried out using AFM1–KLH as an immunogen. One week after each booster injection with AFM1–KLH, the titer and selectivity of antisera were tested for each mouse.

Five injections later, the mouse that produced the highest titer antibody against AFM1 was selected for cell fusion.[19] With four cycles of hybridoma cells, a cell line that steadily produced a specific antibody against AFM1 was selected and expanded. The screened hybridoma cells were injected into mice for ascites production. The ascites were collected and purified by the caprylic acid–ammonium method. OVA and BSA conjugates were used as coating antigens to test the cross-reactivity and sensitivity to AFM1 by ELISA. The affinity constant between antibody and AFM1 was calculated using non-competitive enzyme immunoassay, as previously described.[15]

2.4 Antibody labeled with colloidal gold

Uniform gold nanoparticles scaled at 25 nm were synthesized and characterized by transmission electron microscopy, as described previously.[20] The ultraviolet spectrum indicated the absorption wavelength at 528 nm. Conjugation between the antibody and colloidal gold was carried out as described previously.[21] The resulting conjugates were dissolved in 0.02 M phosphate buffer (containing 5% sucrose, 1% BSA and 0.5% PEG 6000, 0.02% NaN_3, pH 7.4) and stored at 4 °C prior to use.

2.5 Design and assembly of the immunochromatographic test strip

Detection of AFM1 was based on a competitive format.[22] Gold conjugates (antibody against AFM1 labeled with gold nanoparticles) were coated on glass fiber pads. When sample solutions were re-mobilized, the dried conjugates and analytes in the samples interacted with the conjugates, and both migrated into the reaction section of the strip, which was the reaction matrix. These sections were laid down in bands in specific areas of the membrane where they captured the conjugates as they migrated past the capture lines. The results were interpreted in the reaction matrix as the presence or absence of lines of captured conjugate, read either by eye or using a reader, as described previously.[23] In this pattern, antigen of AFM1 and goat anti-mouse IgG were immobilized separately in the strip and the resulting sections were designated the "test line" and "control line". Excess reagents migrated past the capture lines and were entrapped in the wick or absorbent pad.

The coating antigen (0.2 mg mL^{-1} AFB1–BSA) and goat anti-rabbit IgG (0.5 mg mL^{-1}) were sprayed onto NC membranes at 1 μL cm^{-1} using the dispenser to produce the test line and control line on the strip. The treated NC membranes were dried for subsequent use. Similarly, the gold conjugates were sprayed onto glass fiber pads (2 μL mm^{-1}) and then dried for 1 h at 37 °C. The treated NC membranes were pasted on the center of the PVC back plate, and the conjugate, sample and absorbent pads were laminated onto the back plate. The laminate was cut into 4 mm strips and assembled into a plastic cassette.

2.6 Test procedure for the strip

All OVA and BSA conjugates of AFM1 or AFB1 were tested in the "test line" of the strip to optimize the robustness and sensitivity of the AFM1 test strip. Without any dilution, about 100 μL liquid milk (6–8 drops) were loaded on the sample pad and allowed to migrate along the strip under the capillary siphon strength of the absorbance pad. Fifteen minutes later, the depth of red color was assessed by the naked eye.

The assay sensitivity was evaluated with milk samples spiked with various concentrations of AFM1 (0, 0.01, 0.02, 0.05, 0.1, 0.2 and 0.5 μg L^{-1}). Six repeats were done at each level.

One-gram milk powder sample was dissolved in 8 mL water and 100 μL solution was added to the test strip for detection.

The milk powder samples spiked with 0.08–4 μg kg^{-1} AFM1 were tested. The LOD of the strip was defined as the analyte concentration that resulted in the disappearance of the test line.

2.7 Analysis of milk samples

Twenty milk samples including pasteurized milk (10 samples) and ultra-high-temperature (UHT) milk (10 samples) were analyzed with the developed strip. Each sample was detected three times. All results were confirmed by LC/MS-MS by Chinese national standard methods (GB 5413.37).

3 Results and discussion

3.1 Antibody production and characterization

Duo-antigen immunization was carried out in mice. Mice were induced to produce antisera specific for AFM1 using AFM1–KLH immunogen during the second immunization period. AFM1 is a hydroxylated metabolite of AFB1 (Fig. 1). AF derivatives were prepared by conjugation of AFM1 or AFB1 to a protein *via* a carboxymethyloxime (CMO) spacer. The first immunization of AFB1 conjugates was aimed to arouse immune response to AF and the following immunization of AFM1 conjugates would specifically induce the cell clone against AFM1. This strategy was used to avoid possible acquired immunological tolerance during immunization process. The selected cell line 7C6-H1 was verified to secrete monoclonal antibody with heavy chain IgG1 subtype and light chain λ subtype using the Iso-Quick Kit. The affinity constant between AFM1 antibody and AFM1 was 1.28×10^9 mol L^{-1} and the value for AFB1 was 0.76×10^8 mol L^{-1}. The 50% inhibition rate (IC_{50}) of AFM1 was 0.034 ± 0.002 μg L^{-1} using AFB1–BSA as coating antigen while the IC_{50} value was 0.048 ± 0.003 using AFM1–BSA as coating antigen (Table 1). Hence, AFB1–BSA was chosen as coating antigen and the typical inhibition curve was shown in Fig. 2. As shown in Table 1, there was no recognition between 7C6-H1 antibody and other mycotoxins, such as OTA, ZEN or DON. Although the trifle difference between AFM1 and AFB1 lies in the hydroxyl group at the furan ring, the antibody differentiates them well with cross-reaction rates of 3.7–4% for AFB1 (IC_{50} value of 0.910 ± 0.005 μg L^{-1}). The 7C6-H1 antibodies poorly recognized other related AFs (cross-reaction rates less than 1%). All data indicated the good specificity and sensitivity of this monoclonal antibody.

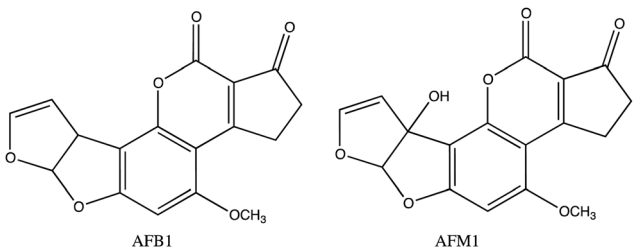

Fig. 1 Chemical structure of aflatoxin (AF) B1 and AFM1.

Table 1 Cross reactivity test of anti-AFM1 monoclonal antibody[a]

	AFB1–BSA coating antigen		AFM1–BSA coating antigen	
Mycotoxins	IC_{50} μg L^{-1}	CR%	IC_{50} μg L^{-1}	CR%
AFM1	0.034 ± 0.002	100.00%	0.048 ± 0.003	100.00%
AFB1	0.910 ± 0.005	3.70%	1.196 ± 0.012	4.01%
AFB2	N	N	N	N
AFG1	>10	<0.34%	>10	<0.48%
AFG2	N	N	N	N
OTA	N	N	N	N
ZEN	N	N	N	N
DON	N	N	N	N

[a] Note: N represents the CR value less than 0.01%.

3.2 Evaluation of the strip test

The blank UHT milk samples spiked with 0.01–0.5 μg L^{-1} AFM1 were evaluated and the results are shown in Fig. 3. The red color of the test line in the strip disappeared completely with liquid milk samples spiked at 0.2 μg L^{-1}, which indicated that the LOD of this strip by the naked eye was 0.2 μg L^{-1} for liquid milk analysis. Similarly, the test line completely disappeared with powdered milk samples spiked at 1.6 μg kg^{-1}, which indicated that the LOD of this strip by the naked eye was 1.6 μg kg^{-1} for the powdered milk analysis.

3.3 Milk sample analysis

Twenty milk samples bought from a local market were analyzed for AFM1 residues and the results were confirmed by GC/MS-MS. No positive samples were found based on the strip analysis. Based on GC/MS-MS analysis, two UHT samples contained AFM1 at 0.013 and 0.008 μg L^{-1}, which was lower than the Chinese maximum residue limit of 0.5 μg L^{-1}. Zheng *et al.*[24] analyzed the presence of AFM1 in 153 UHT milk samples during July and September, 2010 in China. Eighty-four of 153 samples had AFM1 residues, with levels ranging from 0.007 to

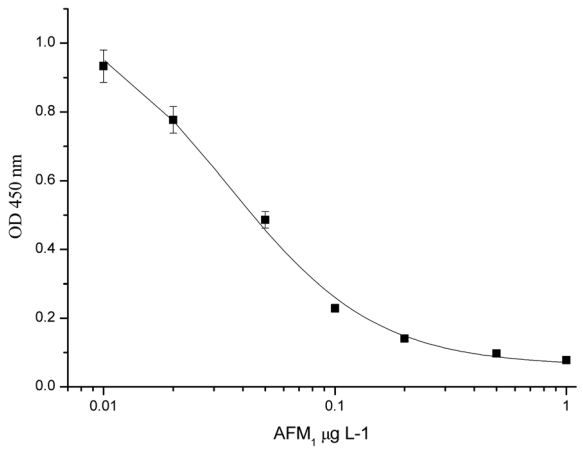

Fig. 2 A typical inhibition curve for the prepared monoclonal antibody.

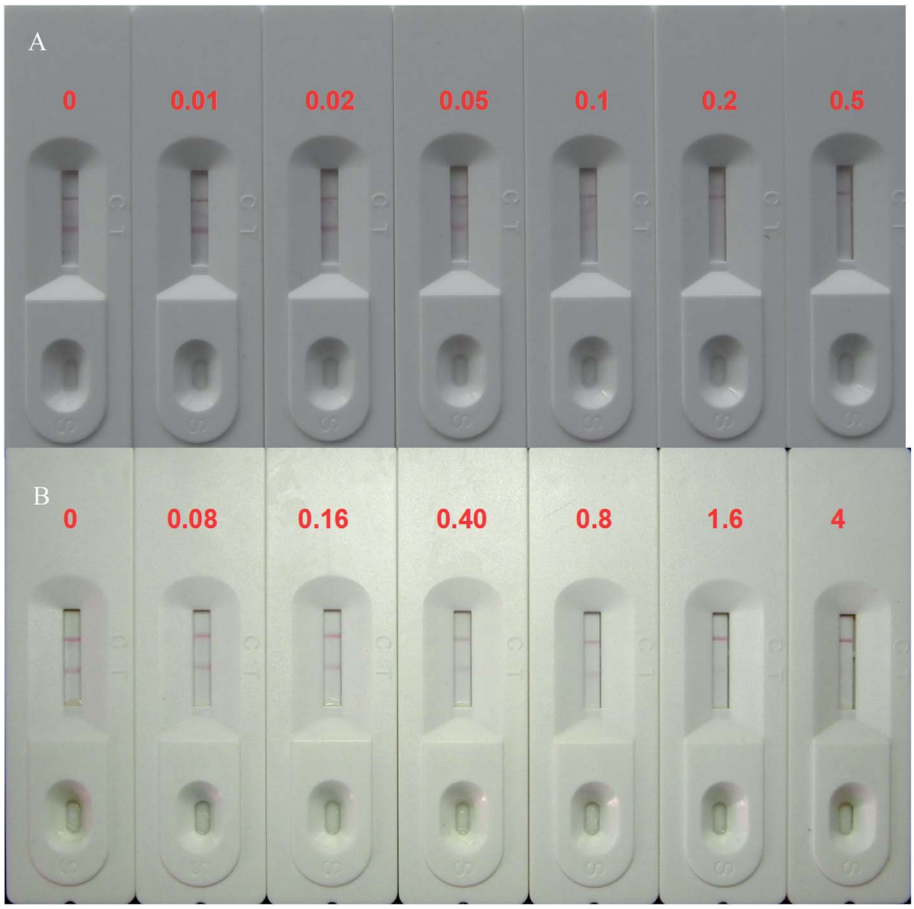

Fig. 3 Sensitivity test of the strip.

0.036 μg L^{-1}. Milk contamination with AFM1 was significantly affected by climatic conditions and geographic location.[25,26] The lower incidence compared with the study of Zheng's may be attributed to the feeding practices of cows and the critical surveillance undertaken by local government.

4 Conclusion

With a high affinity and sensitive antibody, we developed a test strip to directly detect AFM1 in liquid milk. Color disappearance was observed during analysis of standard spiked milk samples and the cut-off value was 0.2 μg L^{-1}. This inexpensive strip avoids complex pretreatment of samples for AFM1 detection and provides a detection threshold lower than the AFM1 tolerance level (0.5 μg L^{-1}) set in China. This indicates that this strip can be used for AFM1 screening of raw milk.

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