Comparison of immunochromatographic assays based on fluorescent microsphere and quantum-dot submicrobead for quantitative detection of aflatoxin M₁ in milk

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ABSTRACT

The performance of fluorescent microsphere immunochromatographic assay (FM-ICA) and quantum-dot submicrobead immunochromatographic assay (QB-ICA) was systematically and comprehensively compared in quantitative detection of aflatoxin M₁ in milk. Under optimum conditions, the advantages of FM-ICA include lower limit of detection of 42.3 pg/mL with better accuracy, precision, reliability, and practicability. The advantages of QB-ICA include shorter detection time and lower monoclonal antibody consumption. The 2 ICA were consistent with liquid chromatography-tandem mass spectrometry. This study serves as a reference for selecting appropriate fluorescent labels for the immunochromatographic assay of aflatoxin M₁.

Key words: immunochromatographic assay, fluorescent microsphere, quantum-dot submicrobead, milk

INTRODUCTION

Aflatoxin M₁ (AFM₁) is a major monohydroxylated derivative of aflatoxin B₁ (AFB₁; Guo et al., 2014), which is produced by cytochrome P450-associated enzymes in the liver and also known as “milk toxin” (Taherimaslak et al., 2014). Aflatoxin M₁ has been classified as a group I carcinogenic agent by the International Agency for Research on Cancer in 2002 (Du et al., 2016). Various analytical methods have been developed to detect AFM₁. These methods include mass spectrometry (Busman et al., 2015), liquid chromatography (Huang et al., 2015), and ultra-high performance

liquid chromatography-tandem mass spectrometry (Ossa et al., 2015). These methods are highly specific and sensitive but require professional operator training and expensive apparatus. Furthermore, the pretreatment of samples is time consuming and unsuitable for on-site detection.

Compared with the above-mentioned methods, immunoassay has attracted attention because of its high sensitivity, high selectivity, simple sample extraction processes, and cost effectiveness (Vdovenko et al., 2014). Immunochromatographic assay (ICA) is a widely used immunoassay. Colloidal gold (CG) is conventionally used as label in ICA because a gold-nanoparticle-based assay is cheap and fast. However, the detection sensitivity of CG-ICA is limited (Shan et al., 2015). In recent years, fluorescent microsphere (FM) and quantum dot (QD) instead of CG have been developed and used in ICA format because of their high sensitivity and accuracy.

Fluorescent microsphere-labeled immunochromatographic assay (FM-ICA) has recently been widely applied in medical diagnosis (Nankoberanyi et al., 2014; Wang et al., 2015; Yang et al., 2015), food safety (Liu et al., 2015), animal health (Huang et al., 2013; Zhang et al., 2016), and agriculture (Chen et al., 2013a; Zhou et al., 2014). Antibodies chemically bind to the surfaces of FM, thus providing a high degree of long-term stability in sample detection. Different from CG-ICA, FM-ICA possesses high sensitivity, good thermal stability, and high FI (Wang et al., 2014).

Quantum dots have attracted great interest as nanoscale fluorescent labels because of their interesting optical and electronic properties, broad UV excitation, narrow fluorescent emission spectra, high quantum yield, large Stoke's shifts, and high photochemical stability (Zhang et al., 2014; Ensafi et al., 2016; Lu et al., 2016). Furthermore, QD submicrobead (QB), in which numerous QD are embedded in a polymer matrix, im-

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prove the sensitivity of immunoassay because of their stronger FI than the corresponding single QD. Many recent studies have used QB-ICA for the ultrasensitive detection of analytes in medical diagnosis (Li et al., 2014; Shen et al., 2015; Hu et al., 2016) and food safety (Duan et al., 2015). They found that QB-ICA has significantly higher sensitivity than the corresponding single QD-ICA.

Xie et al. (2014) and Zhang et al. (2015) compared the use of FM with that of CG in label-based ICA. Meanwhile, Ren et al. (2014) compared the performance of QB with that of the corresponding single QD in label-based ICA. However, a comparison of performance between FM- and QB-based ICA has yet to be conducted. The performance of FM-ICA and QB-ICA in quantitative detection of AFM1 was systematically and comprehensively compared for the first time in the present study. The detection principle based on competitive inhibition is shown in Figure 1. This study serves as a reference for selecting appropriate fluorescent labels for ICA in the detection of AFM1 in milk.

MATERIALS AND METHODS

Materials and Reagents

The FM was purchased from Merck Company (Darmstadt, Germany). The QB was purchased from Ocean NanoTech (San Diego, CA). The AFM1 standard solutions, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (**EDC·HCl**), and BSA were obtained from Sigma-Aldrich (St. Louis, MO). Donkey anti-mouse antibody, anti-AFM1 monoclonal antibody (**mAb**), and AFM1-BSA conjugate with a molar substitution ratio of 40:1 were obtained from Zodolabs Bioengineering Co. Ltd. (Nanchang, Jiangxi, China). Nitrocellulose membrane, absorbent pad, and sample pad were purchased from Millipore (Bedford, MA). Milk samples were purchased from Rainbow Supermarket (Nanchang, Jiangxi, China). All other chemicals and reagents were of analytical grade and purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

Figure 1. Schematic diagram of fluorescent microsphere immunochromatographic assay (FM-ICA) and quantum-dot submicrobead immunochromatographic assay (QB-ICA) for quantitative detection of aflatoxin M_1 (AFM1) in milk. A: Pre-incubation of milk samples and FM/QB-monoclonal antibody (mAb) probe in ELISA well for 10 min. B: Detection principle of ICA strips. NC = nitrocellulose.

Apparatus

The FM-ICA reader (excitation wavelength = 470 nm; emission wavelength = 520 nm) and QB-ICA reader (excitation wavelength = 365 nm; emission wavelength = 620) were from Zhejiang Fenghang Science Instrument Co. Ltd. (Zhejiang, China). The BioDot XYZ platform equipped with a motion controller and Guillotine Cutting Module were supplied by BioDot (Irvine, CA). A vacuum drying oven was purchased from Shanghai Fumma Laboratory Instrument Co. Ltd. (Shanghai, China). A high-speed freezing centrifuge was purchased from Xiang Yi (Hunan, China).

Key Parameters in Optimization of FM-ICA and Preparation of the Test Strip

Preparation of FM-mAb Probe. An FM-mAb probe was prepared as follows. In brief, 6 μg of anti-AFM1 antibody was added to 2.6 mL of 0.01 M phosphate buffer with different pH values (5.0, 6.0, 7.0, 8.0, 9.0, and 10.0) and containing 0.15 mg of FM and 2.7 μL of EDC·HCl (5 mg/mL). The solution was agitated for 2 h. Subsequently, 300 μL of BSA (10%, wt/vol) was added as a blocking buffer, and the solution was agitated for 30 min. After centrifugation ($19,460 \times g$ at 4°C for 10 min), the supernatant was discarded, and the precipitate was resuspended in a 300- μL solution containing 0.02 mol/L of Na_2HPO_4 at pH 5.5, 5% sucrose, 3% trehalose, 0.1% NaN_3 , 1% BSA, and 1% Tween-20. Dynamic light scattering (DLS) analysis of the free FM and FM-mAb probe was performed using the particle size analyzer to confirm the immobilization of anti-AFM1 mAb on the FM surface.

Optimizing the Volume of the FM-mAb Probe. Standard AFM1 milk solutions (98.2, 97.8, and 97.4 μL) were mixed with the FM-mAb probe (1.8, 2.2, and 2.6 μL) in the ELISA well to determine the influence of FM-mAb probe volume on the fluorescent intensity of the T line (FI_T), the fluorescent intensity of the C line (FI_C), $\text{FI}_\text{T}/\text{FI}_\text{C}$, and positive inhibition rate (500 pg/mL). The mixture was then transferred to the FM-ICA strips. After 35 min of immune reaction, the FI_T , FI_C , and $\text{FI}_\text{T}/\text{FI}_\text{C}$ were recorded by the fluorescent strip reader.

Assembling the FM-ICA Test Strip. The AFM1-BSA (0.15, 0.25, and 0.3 mg/mL) and donkey anti-mouse antibody (0.55 mg/mL) were sprayed onto the nitrocellulose membrane to form the T and C lines, respectively. The prepared nitrocellulose membrane and the absorption, conjugate, and sample pads were assembled to prepare the FM-ICA test strip.

Optimization of Key Parameters for QB-ICA and Preparation of Test Strip

Preparation of QB-mAb Probe. A QB-mAb probe was prepared as follows. In brief, 0.5 μg of EDC·HCl, 0.06 mg of QB, and 6 μg of anti-AFM1 mAb were added to 180 μL of 0.01 M phosphate buffer with different pH values (5.0, 6.0, 7.0, 8.0, 9.0, and 10.0). After reacting at room temperature for 1 h under magnetic stirring, the mixtures were centrifuged at $18,070 \times g$ at 4°C for 10 min. The QB and anti-AFM1 mAb conjugates were resuspended with 180 μL of PBS (0.01 M, pH 7.4) containing 2% fructose, 1% polyethylene glycol (PEG 20000, Merck Company), 5% sucrose, 1% BSA, and 0.4% Tween-20. The DLS analysis of the free QB and QB-mAb probe was performed using a particle size analyzer to confirm the immobilization of anti-AFM1 mAb on the QB surface.

Optimizing the Volume of the QB-mAb Probe. Milk samples (79, 78, and 77 μL) were mixed with the QB-mAb probe (1.0, 2.0, and 3.0 μL) in the ELISA well to determine the FI_T , FI_C , $\text{FI}_\text{T}/\text{FI}_\text{C}$, and positive inhibition rate (1,000 pg/mL). The mixture was then transferred to the QB-ICA strips. After 15 min of immune reaction, the FI_T , FI_C , and $\text{FI}_\text{T}/\text{FI}_\text{C}$ were recorded by the fluorescent strip reader.

Assembling the QB-ICA Test Strip. The AFM1-BSA (0.05, 0.1, and 0.15 mg/mL) and donkey anti-mouse antibody (1.0 mg/mL) were sprayed onto the nitrocellulose membrane to form the T and C lines, respectively. The prepared nitrocellulose membrane and the absorption, conjugate, and sample pads were assembled to prepare the QB-ICA strip.

Quantitative Procedure of QB-ICA and FM-ICA

Standard AFM1 milk solutions were prepared using spiked stock AFM1 solution (1.0 $\mu\text{g}/\text{mL}$) in PBS containing 3.0% methanol (vol/vol) at pH 7.4 to final concentrations of 0, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, and 1,250 pg/mL. The FM-mAb probe (2.2 μL) and the QB-mAb probe (1.0 μL) were pre-incubated with 97.8 and 79 μL of standard AFM1 milk solutions in an ELISA well at room temperature to form the FM-mAb probe mixture and QB-ICA mixture, respectively. After 10 min of incubation, the FM-mAb probe mixture and QB-ICA mixture were transferred to FM-ICA and QB-ICA, respectively. The strips were scanned with the fluorescent strip reader. The FI_T , FI_C , and $\text{FI}_\text{T}/\text{FI}_\text{C}$ were recorded. An AFM1 quantitative analysis was calculated according to the linear regression equation of the AFM1 cali-

bration curve. The standard curve was established by plotting B/B_0 against the logarithm of the AFM1 concentration, where B and B_0 represent the FI_T/FI_C with and without AFM1 in the milk solutions, respectively.

Comparison of FM-ICA and QB-ICA

Limit of Detection of FM-ICA and QB-ICA.

The limit of detection (LOD) was calculated as the average FI_T/FI_C of 20 blank milk samples minus 3 standard deviations from the average.

Accuracy and Precision of 2 ICA Strips. The accuracy and precision of FM-ICA were evaluated by analyzing the recovery and coefficients of variation of the intraassays and interassays in 3 spiked milk samples with AFM1 concentrations of 100, 300, and 500 pg/mL. Similarly, the accuracy and precision of QB-ICA were evaluated by analyzing the recovery and coefficients of variation of the intraassays and interassays in 3 spiked milk samples with AFM1 concentrations of 300, 600, and 900 pg/mL. The intraassay was completed within 1 d with 3 replicates for each spiked concentration, and the interassays were continuously conducted every 3 d with 3 replicates for each spiked concentration.

Specificity of FM-ICA and QB-ICA. The specificity of FM-ICA and QB-ICA was evaluated by analyzing AFM1 and 4 other mycotoxins, namely, deoxynivalenol (DON), AFB1, ochratoxin A (OTA), and zearalenone (ZEN) at concentration 10 ng/mL.

Liquid Chromatography-Tandem Mass Spectrometry Analysis. The reliability and practicability of FM-ICA and QB-ICA were further confirmed by a triple quadrupole liquid chromatography-tandem mass spectrometry (LC-MS/MS) system (Agilent Technologies, Lexington, MA) composed of triple quad instruments (Agilent 6410B) and an LC system (Agilent 1200 series). The LC-MS/MS operation was performed in accordance with the national food safety standards of China (GB 5413.37, 2010) with some modifications. A 50-mL milk sample was heated to 35 to 37°C in a water bath and then centrifuged at $8,340 \times g$, at 25°C for 15 min. After centrifugation, all of the supernatant was collected. The treated supernatant was cleaned using an immune affinity column provided by Beijing Rapid Bioscience Co. Ltd. (Beijing, China). The purified AFM1 solution was filtered with a 0.22- μ m cellulose membrane and further used for LC-MS/MS analysis. The LC-MS/MS system was controlled using MassHunter (Agilent Technologies, Lexington, MA). The chromatographic separation was performed with an Agilent Zorbax Eclipse SB-C18 column (2.1 mm $\times$ 100 mm, 1.8 μ m) that was maintained at 35°C. The mobile phase consisted of solvent A (0.1% formic acid solu-

tion) and solvent B (acetonitrile-methanol solution). The gradient elution conditions are as follows: 0 min, 2% solvent B; 0.5 min, 2% solvent B; 3.1 min, 100% solvent B; 6.5 min, 100% solvent B; and 6.2 min, 100% solvent B. The flow rate was 0.30 mL/min, and the injection volume was 10 μ L in full loop injection mode. Electrospray ionization was performed in positive ion mode. Detection was conducted in multi-ion reaction monitoring mode. High-purity nitrogen gas was used for desolvation, as cone, and as collision gas. The gas temperature was 325°C, the gas flow rate was 6 L/min, and the gas pressure was 310,275 Pa. The monitoring ion pairs were chosen as AFM1 m/z 329.1/273.5 (quantitation ions) and 329.1/259.5 (qualitative ions), respectively.

Comparative Evaluation of FM-ICA and QB-ICA with LC-MS/MS

Comparative Evaluation of FM-ICA with LC-MS/MS. The milk sample confirmed as a negative sample was spiked with AFM1 at 3 concentrations (50, 250, and 500 pg/mL) and then analyzed using the developed FM-ICA and LC-MS/MS to study the reliability and practicability of FM-ICA with 3 replicates at each spiked concentration.

Comparative Evaluation of QB-ICA with LC-MS/MS. The milk sample confirmed as a negative sample was spiked with AFM1 at 3 concentrations (200, 500, and 800 pg/mL) and then analyzed using the developed QB-ICA and LC-MS/MS to study the reliability and practicability of QB-ICA with 3 replicates at each spiked concentration.

RESULTS AND DISCUSSION

Detection Principle of ICA

The detection principle of FM/QB-ICA is based on a competitive ICA as shown in the Figure 1. The mixture including milk and probe-antibody complex is transferred to the sample pad of an ICA test strip from the ELISA well. The AFM1 in milk competes with the AFM1-BSA coated on the nitrocellulose membrane for the limited binding sites of the antibody. Then the ICA test strip was detected by ICA reader. The FI_T is inversely proportional to AFM1 concentration in milk.

Transmission Electron Microscopy Images of FM and QB

The size and morphology of FM and QB were studied under a high-resolution transmission electron microscope. Figures 2A, 2B, 2C, and 2D illustrated that the

Figure 2. Transmission electron microscopy (TEM) images of fluorescent microsphere (FM) and quantum-dot submicrobead (QB). A and B: TEM images of FM at different magnifications. C and D: TEM images of QB at different magnifications.

FM and QB presented a narrow size distribution and high monodispersity, with respective average sizes of approximately 175 and 250 nm. Furthermore, Figures 2C and 2D showed a compact QD-polymer structure, indicating that the numerous individual dark dots were tightly encapsulated in the polymer matrix. The dark dots were the QD, which were visibly identified from the polymer matrix because of the different electron penetrabilities between the QD and the polymer matrix.

Fluorescence Spectra of FM and QB

The fluorescence spectra of FM and QB were characterized. Figures 3A and 3B showed that the maximum excitations of FM and QB were at 468 and 395 nm, respectively, whereas their maximized emissions were at 526 and 627 nm, respectively.

DLS of FM, FM-mAb, QB, and QB-mAb

The DLS analysis results (Figures 3C and 3D) showed that the hydrodynamic diameters of the free FM and QB were approximately 206.1 ± 8.2 and 487.3 ± 15 nm, respectively, whereas those of the FM-mAb and QB-mAb probes increased to approximately 242.9 ± 5.2 and 513.6 ± 9.8 nm ($\Delta d_{FM} = 36.8 \pm 3.0$ nm, Δd_{QB}

$= 26.3 \pm 5.2$ nm), respectively, where d = the hydrodynamic diameter, $\Delta d_{FM} = d(\text{FM-mAb}) - d(\text{FM})$, and $\Delta d_{QB} = d(\text{QB-mAb}) - d(\text{QB})$. Results confirmed that the anti-AFM1 mAb was successfully labeled onto the surfaces of the FM and QB.

Effects of Label pH on ICA Sensitivity

Optimizing label pH to develop an ICA is necessary because this parameter can affect antibody activity (Chen et al., 2013b). However, extreme pH exerts adverse effects on antibody activity. The competitive inhibition rate for the 100 pg/mL positive AFM1-spiked milk samples reached a maximum of 48.70% in the pH 7.0 phosphate buffer solution. The results (Figure 4A) indicated that neutral conditions (pH 7.0) improved the sensitivity of FM-ICA by promoting anti-AFM1 mAb and AFM1-BSA recognition. Meanwhile, Figure 4B showed that weak acidic conditions (pH 6.0) improved the sensitivity of QB-ICA by promoting anti-AFM1 mAb and AFM1-BSA recognition.

Kinetic Curves of FM-ICA and QB-ICA

After the FM-ICA and QB-ICA strips were inserted into the FM-ICA and QB-ICA fluorescence readers, respectively, the FI_T , FI_C , and FI_T/FI_C were recorded

Figure 3. Results of fluorescence spectroscopy and dynamic light scattering. (A) Fluorescence spectra of fluorescent microsphere (FM) excitation and emission. (B) Fluorescence spectra of quantum-dot submicrobead (QB) excitation and emission. (C) Dynamic light scattering analysis of the free FM and FM-monovalent antibody (mAb) probe. (D) Dynamic light scattering analysis of the free QB and QB-mAb probe. AFM1 = aflatoxin M₁. Color version available online.

every 1 min total for 40 min. The kinetic curves were constructed by plotting the values of FI_T, FI_C, and FI_T/FI_C against the immune response time. As shown in Figures 4C and 4D, FI_T and FI_C continued to increase. However, the FI_T/FI_C stabilized at 35 min (FM-ICA) and 15 min (QB-ICA).

Optimization of Probe Volume and Concentration of AFM1-BSA Antigen

A similar checkerboard titration was performed with a series of AFM1-BSA concentration on the T line for different volumes of the FM-mAb and QB-mAb probes to achieve the best sensitivity and appropriate FI on both lines. The FM-ICA and QB-ICA results (FI_T, FI_C, and FI_T/FI_C) were recorded at 35 and 15 min, respectively. The competitive inhibition rates were calculated as follows: $(1 - B/B_0) \times 100$, where B₀ and B represent the FI_T/FI_C of the negative sample and AFM1-spiked milk sample (500 pg/mL for FM-ICA and 1,000 pg/

mL for QB-ICA), respectively. As shown in Table 1, the optimal FM-ICA combinations were as follows: 0.25 mg/mL of AFM1-BSA on the T line and 2.2 μL of FM-mAb pre-incubated with 77.8-μL milk samples. Under the optimal conditions, the means of the FI_T and FI_C signals on both lines were $2,984 \pm 33$ and $1,748 \pm 34$, respectively. The competitive inhibition rate for the 500 pg/mL spiked milk sample was $89.70 \pm 0.9\%$. The optimal QB-ICA conditions were as follows: 0.10 mg/mL AFM1-BSA on the T line and 1.0 μL of QB-mAb pre-incubated with 99-μL milk samples. Under the optimal conditions, the means of the FI_T and FI_C signal on both lines were $1,625 \pm 190$ and 446 ± 38 , respectively, and the competitive inhibition rate for the 1,000 pg/mL spiked milk sample was $82.10 \pm 3.4\%$.

The following results were obtained under the optimized conditions of FM-ICA and QB-ICA. Up to 0.05 and 0.01 μg of mAb were consumed in FM-ICA and QB-ICA, respectively; the concentration of AFM1-BSA in FM-ICA was 2.5 times that in QB-ICA.

Figure 4. Optimization of labeling pH and immunoreaction time. A: The effects of labeling pH on the fluorescent intensity of the T line (FI_T)/fluorescent intensity of the C line (FI_C) and positive (100 pg/mL) inhibition ratio of fluorescent microsphere immunochromatographic assay (FM-ICA). B and B₀ represent the fluorescent intensity of the T line/fluorescent intensity of the C line with and without aflatoxin M₁ in the milk solutions, respectively. B: The effects of labeling pH on the FI_T/FI_C and positive (1,000 pg/mL) inhibition ratio of quantum-dot submicrobead immunochromatographic assay (QB-ICA). Error bars indicate SD. C: Immunoreaction dynamics of FI_T, FI_C, and FI_T/FI_C for FM-ICA. a.u. = arbitrary units. D: Immunoreaction dynamics of FI_T, FI_C, and FI_T/FI_C for QB-ICA. Color version available online.

Establishment of the Calibration Curve of FM-ICA and QB-ICA

Under the optimal conditions, a standard curve for the FM/QB-ICA method was constructed by plotting the B/B₀ against the logarithm of AFM1 concentrations. Figure 5A showed that FM-ICA exhibited a dynamic linear range (50–600 pg/mL) with a half-maximal inhibitory concentration (IC₅₀) of 166 pg/mL. Similarly, Figure 5B indicated that QB-ICA exhibited a dynamic linear range (100–1,000 pg/mL) with an IC₅₀ of 349 pg/mL. The 2 regression equations were $y_{\text{FM}} = 1.773 - 0.575 \ln(x)$ ($R^2 = 0.9915$) and $y_{\text{QB}} = 2.394 - 0.753 \ln(x)$ ($R^2 = 0.9903$). The LOD was calculated at 42.3 and 93.2 pg/mL following the method of Anfossi et al. (2013).

The LOD of FM-ICA was approximately 2.2 times lower than that of QB-ICA. The LOD of FM-ICA satisfied the European Union's AFM1 limited standards (50 pg/mL) in milk, whereas the LOD of QB-ICA satisfied

the AFM1 limited standards of America and China (500 pg/mL).

Concentration of FM and QB with the Same FI

The optical intensity of probe is one of the most important parameters for sensitivity of ICA method. To evaluate the FI of the FM and the QB, a Hitachi F-4500 fluorescence spectrophotometer (Tokyo, Japan) was employed to detect the fluorescence intensities of water-soluble FM and QB with the maximum emission wavelength at 526 and 627 nm, respectively. As shown in Figures 6, the FI of FM at concentration of 0.80 nmol/L (dissolved in ultrapure water) was basically the same (about 5,000) as QB at 80.0 nmol/L (dissolved in ultrapure water). Hence, for same number of FM/QB particles, the luminescence intensity of FM was presumably 100 times higher than that of QB. Thus, the fluorescence signal of the FM provides a promising

Figure 5. Results of quantitative detection of fluorescent microsphere immunochromatographic assay (FM-ICA) and quantum-dot submicrobead immunochromatographic assay (QB-ICA). A: Quantitative detection of aflatoxin M₁ (AFM1) by FM-ICA. B: Quantitative detection of AFM1 by QB-ICA. Error bars indicate SD. B and B₀ represent the fluorescent intensity of the T line/fluorescent intensity of the C line with and without AFM1 in the milk solutions, respectively. IC₅₀ = half-maximal inhibitory concentration.

platform to improve the analytical sensitivity of FM-ICA.

Accuracy and Precision of the 2 ICA Strips

The FM-ICA recovery studies of the intraassays and interassays were conducted to evaluate the accuracy

and precision of FM-ICA by analyzing 3 spiked milk samples with low, medium, and high AFM1 concentrations (Table 2). For FM-ICA, the average recoveries for the intraassays ranged from 82.04 to 121.02%, with a CV ranging from 2.48 to 4.73%. The average recoveries for the interassays ranged from 89.13 to 119.08%, with a CV ranging from 4.33 to 6.16%. In QB-ICA, the aver-

Table 1. Results of the checkerboard titration performed to optimize the parameters

No.	Concentration ¹ (mg/mL)	Volume ² (μL)	FI _T ³	FI _C ³	FI _T /FI _C ³	Inhibition rate ⁴
FM						
1	0.15	1.8	1,076 ± 28	745 ± 20	1.444 ± 0.01	79.3 ± 2.0
2	0.15	2.2	1,243 ± 31	1,036 ± 17	1.200 ± 0.01	77.1 ± 2.0
3	0.15	2.6	1,573 ± 74	793 ± 15	1.984 ± 0.05	86.5 ± 3.8
4	0.25	1.8	2,089 ± 54	1,611 ± 14	1.297 ± 0.02	84.2 ± 2.7
5 ⁵	0.25	2.2	2,984 ± 33	1,748 ± 34	1.707 ± 0.01	89.7 ± 0.9
6	0.25	2.6	3,245 ± 95	1,537 ± 52	2.111 ± 0.01	88.4 ± 0.5
7	0.3	1.8	2,577 ± 82	941 ± 35	2.739 ± 0.01	87.4 ± 0.7
8	0.3	2.2	3,270 ± 197	1,236 ± 166	2.646 ± 0.20	86.1 ± 1.0
9	0.3	2.6	3,519 ± 94	958 ± 32	3.673 ± 0.02	82.5 ± 0.1
QB						
1	0.05	1.0	890 ± 218	622 ± 100	1.418 ± 0.14	78.6 ± 2.0
2	0.05	2.0	1,824 ± 142	1,205 ± 180	1.525 ± 0.11	64.2 ± 2.7
3	0.05	3.0	2,409 ± 419	1,703 ± 80	1.409 ± 0.19	39.5 ± 8.8
4 ⁶	0.10	1.0	1,625 ± 190	446 ± 38	3.689 ± 0.78	82.1 ± 3.4
5	0.10	2.0	3,007 ± 266	832 ± 10	3.610 ± 0.28	69.0 ± 2.4
6	0.10	3.0	4,712 ± 718	1,349 ± 116	3.493 ± 0.47	40.9 ± 8.0
7	0.15	1.0	1,695 ± 107	439 ± 26	3.860 ± 0.22	80.9 ± 1.1
8	0.15	2.0	3,352 ± 363	803 ± 161	4.174 ± 0.04	69.7 ± 0.3
9	0.15	3.0	4,845 ± 643	936 ± 76	5.236 ± 1.12	46.4 ± 11.7

¹Aflatoxin M₁ (AFM1)-BSA concentration of fluorescent microsphere immunochromatographic assay (FM-ICA) or quantum-dot submicrobead immunochromatographic assay (QB-ICA).

²Probe volume of FM-monoclonal antibody (mAb) probe or QB-mAb probe.

³FI_T = fluorescent intensity of the T line; FI_C = fluorescent intensity of the C line (±SD).

⁴Inhibition rates (±SD) were obtained from the 500 and 1,000 pg/mL concentrations of AFM1-spiked milk samples for FM-ICA and QB-ICA, respectively.

⁵Optimal parameters of FM-ICA.

⁶Optimal parameters of QB-ICA.

Table 2. Accuracy and precision of the 2 immunochromatographic assay strips¹

Spiked AFM1 (pg/mL)	Intraassay			Interassay		
	Mean $\pm$ SD	Recovery (%)	CV (%)	Mean $\pm$ SD	Recovery (%)	CV (%)
FM						
500	410.2 $\pm$ 14.73	82.04	3.59	445.65 $\pm$ 25.05	89.13	5.62
300	363.07 $\pm$ 9.00	121.02	2.48	357.24 $\pm$ 15.47	119.08	4.33
100	96.87 $\pm$ 4.58	96.87	4.73	97.06 $\pm$ 5.98	97.06	6.16
QB						
900	707.30 $\pm$ 72.07	78.56	10.19	768.01 $\pm$ 74.73	85.33	9.73
600	537.99 $\pm$ 17.05	89.67	3.17	568.84 $\pm$ 21.90	94.81	3.85
300	218.98 $\pm$ 22.88	73.00	10.45	350.42 $\pm$ 33.50	116.81	9.56

¹AFM1 = aflatoxin M₁; FM = fluorescent microsphere; QB = quantum-dot submicrobead.

age recoveries for the intraassay and interassay were 73.00 to 89.67% and 85.33 to 116.81%, respectively, with corresponding CVs of 3.17 to 10.45% and 3.85 to 9.73%.

Reliability and Practicability of FM-ICA and QB-ICA

To evaluate the reliability and practicability of FM-ICA and QB-ICA, the 2 methods were compared with LC-MS/MS. Negative milk samples spiked with AFM1 at 50, 250, and 500 pg/mL were tested using FM-ICA and LC-MS/MS. Negative milk samples spiked with AFM1 at 200, 500, and 800 pg/mL were tested using QB-ICA and LC-MS/MS.

As shown in Table 3, the linear correlation equation between FM-ICA and LC-MS/MS was $y = 1.08x - 1.43$ with a reliable correlation coefficient ($R^2 = 0.994$), and the linear correlation equation between QB-ICA and LC-MS/MS was $y = 0.89x + 35.52$ with a reliable correlation coefficient ($R^2 = 0.967$).

Specificity of FM-ICA and QB-ICA

To evaluate their specificity, FM-ICA and QB-ICA were tested with AFM1 and 4 aflatoxins, namely, DON,

AFB1, OTA, and ZEN at concentrations of 10 ng/mL. As shown in Figure 7, the FI_T/FI_C of DON, OTA, and ZEN were as high as those of the negative samples. This result indicated that FM-ICA and QB-ICA lack immune responses to DON, OTA, and ZEN. However, the FI_T/FI_C of AFB1 was 0 because AFM1 is a major monohydroxylated derivative of AFB1. Thus, FM-ICA and QB-ICA can specifically detect AFM1.

Validation of the Methods for Practical Use

The 12 raw milk samples were detected using FM-ICA, QB-ICA, and LC-MS/MS. The result was presented in Table 4. The concentration of AFM1 in 12 milk samples was lower than the LOD of FM-ICA and QB-ICA. The detection results of the FM-ICA and QB-ICA were in good agreement with that of the LC-MS/MS method.

CONCLUSIONS

Systematic and comprehensive comparison between novel FM-ICA and QB-ICA in quantitative detection of AFM1 in milk was studied for the first time. The advantages of FM-ICA include lower LOD of 42.3 pg/mL,

Table 3. Correlation between results from (x-axis) liquid chromatography-tandem mass spectrometry (LC-MS/MS) and quantum-dot submicrobead immunochromatographic assay (QB-ICA)/fluorescent microsphere immunochromatographic assay (FM-ICA; y-axis) analyses of aflatoxin M₁ (AFM1) in spiked milk samples

Spiked AFM1 (pg/mL)	ICA results			LC-MS/MS		
	Mean ¹ (pg/mL)	Recovery (%)	CV (%)	Mean (pg/mL)	Recovery (%)	CV (%)
FM						
50	37.89 $\pm$ 3.46	75.78	9.13	38.90 $\pm$ 5.10	77.81	10.21
250	217.75 $\pm$ 9.65	87.10	4.43	201.60 $\pm$ 11.19	81.39	4.47
500	451.20 $\pm$ 11.9	90.24	2.64	419.34 $\pm$ 33.14	83.87	6.63
QB						
200	159.62 $\pm$ 7.23	79.81	4.53	167.45 $\pm$ 19.65	81.73	9.83
500	451.20 $\pm$ 11.90	90.24	2.38	419.34 $\pm$ 33.14	83.87	6.63
800	620.24 $\pm$ 35.73	77.53	5.76	703.49 $\pm$ 29.13	87.94	3.64

¹Mean ($\pm$ SD) value of 3 replicates at each spiked concentration.

Figure 6. Photoluminescence spectra of fluorescent microsphere (FM; 0.80 nmol/L, dissolved in ultrapure water) and quantum-dot submicrobead (QB; 80.0 nmol/L, dissolved in ultrapure water). a.u. = arbitrary units. Color version available online.

better accuracy and precision, and better reliability and practicability. The advantages of QB-ICA include short detection time and low mAb consumption. This study serves as a reference for selecting appropriate fluorescent labels for the ICA of AFM1 in milk.

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Table 4. Detection results of raw milk samples¹

Sample	Detection results (pg/mL)		
	FM-ICA	QB-ICA	LC-MS/MS
1	ND	ND	ND
2	ND	ND	ND
3	ND	ND	ND
4	ND	ND	5.12
5	ND	ND	3.04
6	ND	ND	ND
7	ND	ND	ND
8	ND	ND	ND
9	ND	ND	ND
10	ND	ND	0.86
11	ND	ND	1.16
12	ND	ND	ND

¹FM-ICA = fluorescent microsphere immunochromatographic assay; QB-ICA = quantum-dot submicrobead immunochromatographic assay; LC-MS/MS = liquid chromatography-tandem mass spectrometry; ND = not detected.

Figure 7. Specificity experiment of fluorescent microsphere immuno-chromatographic assay (FM-ICA) and quantum-dot submicrobead immuno-chromatographic assay (QB-ICA) for 10 ng/mL of aflatoxin M₁ (AFM1), deoxynivalenol (DON), aflatoxin B₁ (AFB1), ochratoxin A (OTA), and zearalenone (ZEN). Error bars indicate SD. Color version available online.

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