Lateral flow immunoassay integrated with competitive and sandwich models for the detection of aflatoxin M₁ and *Escherichia coli* O157:H7 in milk

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ABSTRACT

Pathogens, mycotoxins, or antibiotics may exist in a food sample. Micro- and macromolecular substances must be detected quickly. A rapid and convenient lateral flow immunoassay (LFI) integrated with competitive and sandwich models was developed to detect micro- and macromolecular substances. In this study, aflatoxin M₁ (AFM₁) and *Escherichia coli* O157:H7 were selected as the micro- and macromolecular substances, respectively. Two test lines in the LFI test strip were evaluated to detect AFM₁ and *E. coli* O157:H7 by competitive and sandwich models. Results showed that the limits of detection for detecting AFM₁ and *E. coli* O157:H7 were 50 pg·mL⁻¹ and 1.58 × 10⁴ cfu·mL⁻¹, respectively. The whole assay time was 30 min. The recoveries of gold nanoparticle-LFI ranged from 78.0 to 111.6% with coefficients of variation in the range of 3.9 to 8.5% for the detection of AFM₁. For the detection of *E. coli* O157:H7, the range of recoveries was from 70.1 to 89.6% with coefficients of variation ranging from 4.9 to 13.0%. This study not only tested sensitivity and specificity, but also was a systematic study of location of 2 test lines of the LFI test strip integrated with competitive and sandwich models.

Key words: lateral flow immunoassay, competitive and sandwich model, aflatoxin M₁, *Escherichia coli* O157:H7

INTRODUCTION

Food safety is an important concern and has been paid considerable attention worldwide. Common problems on food safety include foodborne pathogens (Mandal et al., 2011; Shan et al., 2015), mycotoxins (Kabak et al., 2006; Burmistrova et al., 2014), metal ions (Daşbaşı et al., 2016; Gouda, 2016), and antibiotics (Taranova et al.,

2015; Tlili et al., 2016). Several hazardous substances may exist in one food sample. Various methods, such as liquid chromatography-tandem mass spectrometry (LC-MS/MS; Beltrán et al., 2011; Zhang et al., 2016) and conventional ELISA (Urusov et al., 2015; Huang et al., 2017), have been reported for the detection of these compounds. However, conventional ELISA requires professional operation, whereas LC-MS/MS is not suitable for onsite testing because of the size and weight of the equipment and its need for a power supply.

Lateral flow immunoassay (LFI), which is time saving, easy to use, low cost, and user friendly (Song et al., 2014; Chapman et al., 2015; Shin and Park, 2016), has been applied to detect foodborne pathogens (Xie et al., 2014), mycotoxins (Wang et al., 2011; Liu et al., 2015), metal ions (Fang et al., 2010), and antibiotics (Peng et al., 2016b). Competitive and sandwich assays are the 2 main models in LFI. The competitive assay is more suitable if the targets are of low molecular weight or present a single specific antigen. Assays for the simultaneous detection of micromolecular substances based on the competitive method have been reported. Chen et al. (2016) simultaneously detected 3 mycotoxins: aflatoxin B₁ (AFB₁), zearalenone (ZEN), and ochratoxin A (OTA) in food samples. Peng et al. (2016a) detected 32 quinolones using one monoclonal antibody. Sandwich assay is performed when targets exhibit several epitopes. Simultaneous testing of macromolecules based on sandwich method has also been reported. Lee et al. (2016) used 2-color lateral flow assay for multiplex detection of Dengue and Chikungunya viruses. Zhao et al. (2016) rapidly and simultaneously detected 10 foodborne pathogens using a 10-channel lateral flow assay.

Foodborne pathogens (such as *Escherichia coli* O157:H7), mycotoxins (such as aflatoxin M₁, AFM₁), and antibiotics may exist in milk. Foodborne pathogens are macromolecule substances, whereas mycotoxins and antibiotics are micromolecule substances. Thus, it is necessary to detect micro- and macromolecule substances simultaneously.

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Aflatoxin M₁ is a hydroxylated metabolite of AFB₁. Under humid conditions, AFB₁ can emerge in human foodstuffs through direct contamination of cereals. Dairy cows which were fed AFB₁-contaminated feeds may produce the milk contained AFM₁. Aflatoxin M₁ is also known as “milk toxin” (Guo et al., 2014; Taherimaslak et al., 2014). Aflatoxin M₁ has been classified as a group I carcinogenic agent by the International Agency for Research on Cancer (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2002). *Escherichia coli* O157:H7 is one of the most harmful foodborne pathogens that can cause serious illnesses, even death (Wu et al., 2015). *Escherichia coli* O157:H7 may exist in raw milk (Miszczycha et al., 2016). In this study, AFM₁ (328, MW) and *E. coli* O157:H7 were selected as micro- and macromolecular substances, respectively, to be detected by LFI. To our knowledge, Qi et al. (2016) first used LFI integrated with competitive and sandwich models to simultaneously detect prolactin and C-reactive protein. In this paper, both antigens were macromolecules (protein). However, the current study is a systematic investigation using LFI integrated with competitive and sandwich models to detect micro- and macromolecular substances (Figure 1). The location of 2 test lines was important and interesting in the system and was systematically studied.

MATERIALS AND METHODS

Materials and Reagents

The HAuCl₄·3H₂O, trisodium citrate, BSA, and standard solutions of AFM₁, deoxynivalenol (DON), OTA, ZEN, and AFB₁ were purchased from Sigma-Aldrich

Chemical Co. (St. Louis, MO). The polyethylene glycol (PEG) 20,000 was purchased from Merck Co. (Darmstadt, Germany). The nitrocellulose (NC) membrane, absorbent pad, and sample pad were purchased from Millipore (Bedford, MA). Goat anti-mouse antibody, anti-*E. coli* O157:H7-monoclonal antibody (mAb), anti-AFM₁-mAb (half-maximal inhibitory concentration, IC₅₀, is 300 pg·mL⁻¹), rabbit anti-*E. coli* O157:H7-polyclonal antibody (pAb), and AFM₁-BSA conjugate with molar substitution ratio of 40:1 were obtained from Zedolabs Bioengineering Co. Ltd. (Nanchang, Jiangxi, China). Raw milk samples were purchased from Rainbow Supermarket (Nanchang, China). All other chemicals and reagents were of analytical grade and purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

A portable gold nanoparticle (GNP) reader (HELMEN) was purchased from Zhejiang Fenghang Science Instrument Co., Ltd. (Zhejiang, China). The BioDot XYZ platform equipped with a motion controller was 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).

Bacterial Strain Culturing

Escherichia coli O157:H7 (ATCC 43888) and 18 other (non-*E. coli* O157:H7) strains were used in the study. The 18 non-*E. coli* O157:H7 were *E. coli* O45 (ATCC BAA2193), *E. coli* O121 (ATCC BAA2119GFP), *E. coli* O26 (ATCC BAA2443), *Staphylococcus aureus* (CMCC 26003), *Enterobacter sakazakii* (CMCC 45407), *Shigella*

Figure 1. Lateral flow test strip integrated with sandwich and competitive model. Color version available online.

flexneri (CMCC 2457), *Shigella sonnei* (CMCC 51592), *Candida albicans* (ATCC 10231), *Bacillus subtilis* (BD 168), *Listeria welshimeri* (ATCC 35897), *Proteus bacillus vulgaris* (CMCC 49027), *Pseudomonas aeruginosa* (CMCC 11997), *Micrococcus luteus* (CMCC 28001), *Salmonella* Typhimurium (ATCC 13311), *Salmonella* Choleraesuis (ATCC 10708), *Salmonella* Enteritidis (ATCC 13076), *Salmonella* Paratyphi A (ATCC 9150), and *Salmonella* Anatis (ATCC 9270).

Preparation of GNP

The GNP were prepared according to a method provided in a previous report (Huang and Kim, 2011). Briefly, 99 g of ultrapure water was heated to boiling in an oil bath. Then, 1 mL of 1% (wt/vol) HAuCl₄ solution was added into the boiling water under vigorous stirring. After 3 min, 1.45 mL of 1% sodium citrate solution was added into the previous solution with continued boiling. After 30 min, the color of the boiled solution changed to ruby red, indicating the formation of AuNP. The solution was cooled to room temperature and stored at 4°C.

Preparation of LFI Test Strip

The sample pad and NC membrane were pretreated according to previous methods (Luo et al., 2017). 0.74 $\mu\text{L cm}^{-1}$ of AFM₁-BSA (1.0 mg·mL⁻¹) and anti-*E. coli* O157:H7-pAb (0.8 mg mL⁻¹) were sprayed on the NC membrane as test (T) line by using the BioDot XYZ platform. Goat anti-mouse IgG (0.7 mg·mL⁻¹) 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 test strip.

Optimization of Immunomodified GNP Production

In accordance with a previous report (Xie et al., 2014), the GNP solution was adjusted to pH 5.0, 6.0, 7.0, 8.0, and 9.0 with 0.2 M K₂CO₃. Then, under gentle stirring, 1.0, 2.0, 3.0, 4.0, and 5.0 μL of anti-*E. coli* O157:H7-mAb (2.0, 4.0, 6.0, 8.0, and 10.0 $\mu\text{g mL}^{-1}$) or 1.4, 1.7, 2.0, 2.3, and 2.6 μL of anti-AFM₁-mAb (2.0, 2.5, 3.0, 3.5, and 4.0 $\mu\text{g mL}^{-1}$) were added dropwise to 1 mL of the GNP solution to form the GNP-mAb complex. After agitating for 60 min, the complex was sequentially blocked with 100 μL of 1% (wt/vol) PEG 20,000 solution for 30 min and 100 μL of 10% (wt/vol) BSA solution for 30 min. Finally, the mixture was centrifuged at $12,107 \times g$ at 4°C for 30 min. The pellet was resuspended in 100 μL of protein-nanoparticles protection buffer solution, which contained 5% sucrose,

2% trehalose, 1% PEG 20,000, 1% BSA, and 0.25% Tween 20, and stored in an ELISA well at 4°C.

Immunoassay Procedure

As shown in Figure 1, 1 μL of anti-AFM₁-mAb-GNP and 4 μL of anti-*E. coli* O157:H7-mAb-GNP were incubated with 100 μL of PBS or milk samples (0.1 mL of *E. coli* O157:H7 or AFM₁ was added into 1 mL of diluted milk, which was diluted 5 times with 0.01 M PBS (Mayer et al., 1996) and then was mixed for 1 min before strip assay) in an ELISA well at room temperature for 5 min. Then, 100 μL of the mixture was pipetted to the sample pad of the test strip. The signals of T line 1 (T₁) and T line 2 (T₂) were recorded by the GNP reader. The GNP reader could record the absorbance of GNP captured on the T line and C line. Colloidal gold exhibits strong absorbance in a high-power 525 nm light-emitting diode, which was used as light emitter. An optical lens was applied to focus the light source on the strip. The reflective optical signal from the strip was focused using a pair of optical lens and converted to photoelectrical signals. The signals of the T line and C line were proportional to the amount of colloidal gold captured on the lines (Li et al., 2013).

Optimal Location of 2 Test Lines

The LFI setup I was the format of T₁ and T₂ that was used to detect AFM₁ and *E. coli* O157:H7, respectively; LFI setup II was the format of T₁ and T₂ that was used to detect *E. coli* O157:H7 and AFM₁, respectively (Figure 2). In LFI setup I and II, the signal of the T line for detecting *E. coli* O157:H7 (3.3×10^5 cfu·mL⁻¹) was recorded every minute until 35 min when the concentration of AFM₁ was 0 and 500 pg·mL⁻¹, respectively. Then, the signals of the T line (*E. coli* O157:H7) in the 2 assays were compared. In LFI setup I and II, the signal of T line for detecting AFM₁ (0 and 500 pg·mL⁻¹ respectively) was recorded every minute until 35 min when the *E. coli* O157:H7 concentration was 0 and 3.3×10^5 cfu·mL⁻¹, respectively. The signals of T line (*E. coli* O157:H7) in the 2 assays were compared.

To detect *E. coli* O157:H7, the signal of the T line was used to analyze the results. To detect AFM₁, the competitive inhibition ratio was obtained by $1 - T/T_0$ to analyze the results, where T and T₀ were the signals of the T line of negative and positive samples, respectively (Kong et al., 2016).

Data Analysis

First, the signals of the T line for the detection of AFM₁ and *E. coli* O157:H7 in diluted milk were re-

Figure 2. Lateral flow immunoassay (LFI) setup I and LFI setup II formats. AFM₁ = aflatoxin M₁. Color version available online.

corded every minute until 40 min. The immunological kinetic curves were established by plotting the signal intensity of the T line against time.

Then, the standard competitive inhibition curves were gained by plotting $1 - T/T_0$ against the concentration of AFM₁ (0, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1,000 pg·mL⁻¹) in the diluted milk. A standard calibration curve was established by plotting the logarithm of the concentration of *E. coli* O157:H7 (0, 1.65×10^2 , 3.3×10^2 , 1.65×10^3 , 3.3×10^3 , 1.65×10^4 , 3.3×10^4 , 1.65×10^5 , 3.3×10^5 , 1.65×10^6 , and 3.3×10^6 cfu·mL⁻¹) in the diluted milk.

Finally, the spiked samples (250, 450, and 600 pg·mL⁻¹) were detected with the test strips to verify the recovery of AFM₁. The spiked samples (5.0×10^4 , 1.0×10^5 , and 6.0×10^5 cfu·mL⁻¹) were detected with the test strips to verify the recovery of *E. coli* O157:H7. Each concentration was then analyzed in 3 replicates.

RESULTS AND DISCUSSION

Principle

The detection principles are shown in Figure 1. The anti-*E. coli* O157:H7-GNP and anti-AFM₁-GNP were

incubated with samples in ELISA wells at room temperature for 5 min. The sample was then pipetted in the sample pad of the test strip. If *E. coli* O157:H7 exists in the sample, the anti-*E. coli* O157:H7-GNP binds with *E. coli* O157:H7 to form the GNP-mAb-*E. coli* O157:H7 immunocomplex, which is then captured by the anti-*E. coli* O157:H7-pAb to form a signal on T1. The mechanism of the sandwich format is that the signal on T1 line is proportional to the amount of *E. coli* O157:H7. If AFM₁ exists in the sample, the anti-AFM₁-GNP binds with AFM₁ to form the GNP-mAb-AFM₁ immunocomplex, which is not captured by AFM₁-BSA on T2. The AFM₁ is detected through a competition format. The mechanism of the competition format is that the signal on T2 line is inversely proportional to the amount of AFM₁. Two GNP-mAb probes were captured by goat anti-mouse IgG to form signals on the control lines.

As shown in Figure 3A, T1 showed an obvious red line, whereas T2 did not when the concentrations of *E. coli* O157:H7 and AFM₁ were 3.3×10^5 cfu·mL⁻¹ and 500 pg·mL⁻¹, respectively. However, when the concentrations of *E. coli* O157:H7 and AFM₁ were 0 cfu·mL⁻¹ and 0 pg·mL⁻¹, respectively, T1 did not show an obvious red line, whereas T2 did (Figure 3D). No obvious red line developed in T1 and T2 when the concentrations of *E. coli* O157:H7 and AFM₁ were 0 cfu·mL⁻¹ and 500 pg·mL⁻¹, respectively (Figure 3B). Obvious red lines developed in T1 and T2 when the concentrations of *E. coli* O157:H7 and AFM₁ were 3.3×10^5 cfu·mL⁻¹ and 0 pg·mL⁻¹, respectively (Figure 3C).

Characterization of GNP and GNP Probes

The transmission electron micrographs showed that the GNP were 24 nm in diameter (Supplemental Figure S1A; <https://doi.org/10.3168/jds.2018-14655>). As shown in Figure S1B (<https://doi.org/10.3168/jds.2018-14655>), the characterized absorption peaks of GNP, GNP-AFM₁-mAb, and GNP-*E. coli* O157:H7-mAb were 524, 530, and 530 nm, respectively. The curves of the 2 kinds of GNP-mAb showed a red shift compared with those of the GNP because of the mAb on the GNP surface.

Immunological Kinetics Analysis of LFI

Immunological kinetic curve between anti-AFM₁-mAb-GNP and AFM₁-BSA on the T line was established by plotting the signal of the T line when the concentrations of AFM₁ were 0, 500, and 1,000 pg·mL⁻¹. Immunological kinetic curve was established by plotting the signal of T line to detect *E. coli* O157:H7 when the concentrations of *E. coli* O157:H7 were 3.3×10^4 , 3.3×10^5 , and 3.3×10^6 cfu·mL⁻¹. In Supplemental Figure S2

Figure 3. Test strips tested with analytes of different concentrations: (a) aflatoxin M₁ (AFM₁; 500 pg·mL⁻¹) and *Escherichia coli* O157:H7 (3.3×10^5 cfu·mL⁻¹), (b) AFM₁ (500 pg·mL⁻¹) and *E. coli* O157:H7 (0 cfu·mL⁻¹), (c) AFM₁ (0 pg·mL⁻¹) and *E. coli* O157:H7 (3.3×10^5 cfu·mL⁻¹), and (d) AFM₁ (0 pg·mL⁻¹) and *E. coli* O157:H7 (0 cfu·mL⁻¹). C = control line; T 1 = test line 1; T 2 = test line 2. Color version available online.

(<https://doi.org/10.3168/jds.2018-14655>), 23 min was selected as the final quantitative detection of AFM₁ and *E. coli* O157:H7.

Optimization of Immunomodified GNP Production

A series of optimization steps was performed to improve the limit of detection (LOD) of the LFI. When the pH of GNP coupling with anti-AFM₁-mAb was 5.0, the signals of the T line of the negative and positive samples were 539 and 144, respectively, and the inhibition rate was 0.73 (Figure 4A). At pH 6.0, the signals of the T line of the negative and positive samples were 683 and 168, respectively, and the inhibition rate was 0.75. Therefore, pH 6.0 was regarded as the optimal pH for GNP coupling with anti-AFM₁-mAb. Figure 4B shows that 6.0 was the optimal pH for GNP coupling with anti-*E. coli* O157:H7-mAb because the strongest and lowest signals (1271 and 89) of the T line appeared for the detection of the positive and negative samples, respectively.

The concentration of mAb coupling with the GNP was also evaluated. When the concentrations of the anti-AFM₁-mAb were 2.0 and 2.5, the signals of the T line of the negative sample were too weak to be observed by the naked eye [250 and 500 arbitrary units (AU),

Figure 4C]. When the concentrations of the anti-AFM₁-mAb were 3.0, 3.5, and 4.0 μg·mL⁻¹, the signals of the T line of the positive sample were 195, 173, and 1,255 AU, respectively, whereas those of the negative sample were 886, 1,093, and 1,768 AU, respectively (Figure 4C). When the concentrations of anti-AFM₁-mAb were 3.0, 3.5, and 4.0 μg·mL⁻¹, the inhibition rates were 0.78, 0.84, and 0.29, respectively. The highest inhibition rate was 0.84, so 3.5 μg·mL⁻¹ was regarded as the optimal concentration of anti-AFM₁-mAb for coupling with GNP. When the concentration of mAb for coupling GNP was from 2.0 to 4.0 μg·mL⁻¹, the signal of the T line of the positive sample was significantly improved (171 to 1,500 AU). However, when the concentration of anti-*E. coli* O157:H7-mAb for coupling GNP was over 4.0 μg·mL⁻¹, the signal of the T line of the positive sample was almost the same. Thus, 4.0 μg·mL⁻¹ was selected as the optimal concentration of anti-*E. coli* O157:H7-mAb for coupling with GNP (Figure 4D).

Analysis of LFI Setups I and II

To verify that anti-AFM₁-mAb-GNP and anti-*E. coli* O157:H7-mAb-GNP did not interfere with the detection of *E. coli* O157:H7 and AFM₁, respectively, intra-specificity assay was performed. As shown in Figure

Figure 4. Optimization of parameters. (A) Optimization of coupling pH (5, 6, 7, 8, and 9) between gold nanoparticle (GNP) and anti-aflatoxin M₁ (AFM₁)-monoclonal antibody (mAb). (B) Optimization of coupling pH (5, 6, 7, 8, and 9) between GNP and *E. coli* O157:H7-mAb. (C) Optimization of the concentration of anti-AFM₁-mAb conjugating to the GNP (2, 2.5, 3, 3.5, and 4 µg·mL⁻¹). (D) Optimization of the concentration of anti-*E. coli* O157:H7-mAb conjugating to the GNP (2, 4, 6, 8, and 10 µg·mL⁻¹). Data were based on 3 replicates. T and T₀ were the signals of the T line of negative and positive samples, respectively (Kong et al., 2016). Error bars represent SD (n = 3).

5A, when only anti-AFM₁-mAb-GNP existed in the ELISA well, no signal appeared on T1 (1–3). Similarly, T2 signal also did not develop (Figure 5, 4–6) when only anti-*E. coli* O157-mAb existed in the ELISA well. Furthermore, to learn more about the details of the interference between the 2 targets, we prepared the strip with single T line and the strip with 2 analytical lines, tested 2 strips with a single target, and compared their sensitivity. As shown in Figure 5B and Figure 5C, compared the sensitivity of strip with only single line for detection of *E. coli* O157:H7, the sensitivity of strip with 2 analytical lines had no significant changes after the addition of AFM₁. Supplemental Figure S3 (<https://doi.org/10.3168/jds.2018-14655>) showed that the sensitivity of 2 strips had no significant changes for detection of AFM₁ after the addition of *E. coli* O157:H7. Therefore, interference and cross-binding were not present between the 2 sets of biocomponents.

The location of the 2 test lines on the strip should be considered because the LOD of the LFI test strip may

be markedly influenced by the location of these lines. As shown in Figures 6A and 6B, whether the concentration of AFM₁ was 0 or 500 pg·mL⁻¹, the signal for the detection of *E. coli* O157:H7 in LFI setup II was stronger than that in LFI setup I within 35 min. For example, in the Figure 6A, the signal (*E. coli* O157:H7) in LFI setup II (852.5, 4.2% of CV) was significantly stronger than that in LFI setup I (665, 5.4% of CV) at 23 min when the concentration of AFM₁ was 0 pg·mL⁻¹ ($P < 0.05$). It is interesting that (Figures 6C and 6E), whether the concentration of *E. coli* O157:H7 was 0 or 3.3×10^5 cfu·mL⁻¹, the signal for the detection of AFM₁ (0 pg·mL⁻¹) in LFI setup I was stronger than that in LFI setup II within 35 min. For example, in the Figure 6E, the signal (AFM₁) in LFI setup I (811, 1.2% of CV) was stronger than that in LFI setup II (749, 1.3% of CV) at 23 min when the concentration of *E. coli* O157:H7 was 3.3×10^5 cfu·mL⁻¹ ($P < 0.05$). Figures 6D and 6F demonstrate that at 0 or 3.3×10^5 cfu·mL⁻¹ *E. coli* O157:H7 concentration, the signal for AFM₁

detection ($500 \text{ pg} \cdot \text{mL}^{-1}$) in LFI setup I was stronger than that in LFI setup II within 35 min. For example, in the Figure 6F, the signal (AFM₁) in LFI setup II

Figure 5. Intra-specificity analysis. (A) Anti-*Escherichia coli* O157:H7-monovalent antibody (mAb)-gold nanoparticle (GNP) was absent and anti-aflatoxin M₁ (AFM₁)-mAb-GNP was present (1–3). Anti-AFM₁-mAb-GNP was absent and anti-*E. coli* O157:H7-mAb-GNP was present (4–6). Concentrations of AFM₁ and *E. coli* O157:H7 were $0 \text{ pg} \cdot \text{mL}^{-1}$ and $3.3 \times 10^3 \text{ cfu} \cdot \text{mL}^{-1}$, respectively. (B) The sensitivity of the strip with only single test (T) line for *E. coli* O157 (0, 3.3×10^3 , 3.3×10^4 , 3.3×10^5 , and $3.3 \times 10^6 \text{ cfu} \cdot \text{mL}^{-1}$ from left to right). (C) The sensitivity of the strip with 2 analytical lines for *E. coli* O157 (0, 3.3×10^3 , 3.3×10^4 , 3.3×10^5 , and $3.3 \times 10^6 \text{ cfu} \cdot \text{mL}^{-1}$ from left to right). The concentration of AFM₁ was $50 \text{ pg} \cdot \text{mL}^{-1}$. C = control line; T1 = test line 1; T2 = test line 2. The arrows indicate the cutoff value for detection of *E. coli* O157:H7. Color version available online.

(320, 1.6% of CV) was weaker than that in LFI setup I (352, 2.5% of CV) at 23 min when the concentration of *E. coli* O157 was $3.3 \times 10^5 \text{ cfu} \cdot \text{mL}^{-1}$ ($P < 0.05$). These results (Figures 6A–6F) showed that whether the model was a sandwich or competitive model, the signal of the T line close to the sample pad was stronger than that away from the sample pad. This was unsurprising as the anti-*E. coli* O157:H7-pAb or AFM₁-BSA on the test line close to the sample pad could capture many immunocomplexes and exhibit strong signals.

However, it is obvious that the inhibition rate for the detection of AFM₁ in LFI setup I was lower than that in LFI setup II (Figures 6G and 6H). For example, in the Figure 6G, the inhibition (AFM₁) in LFI setup II (0.45, 1.3% of CV) was higher than that in LFI setup I (0.39, 1.5% of CV) at 23 min when the concentration of *E. coli* O157 was $0 \text{ cfu} \cdot \text{mL}^{-1}$ ($P < 0.05$). Presumably, compared with the reaction time between the anti-AFM₁-mAb-GNP probe and AFM₁-BSA on the T line, the T line (AFM₁-BSA) in the LFI setup II assay was away from the sample pad, resulting in longer immunoreaction time between the anti-AFM₁-mAb-GNP probe and AFM₁ in the sample. Consequently, less anti-AFM₁-mAb-GNP probes were captured by the T line, resulting in higher inhibition rate. In a systematical study, we found that if the sandwich model coexists with the competitive model, the sandwich model should be close to the sample pad, and the competition model should be away from the sample pad (LFI setup II; Figure 1). The interesting result could be used in practical detection for improving the LOD of micro- and macromolecular substances at the same time.

LOD of LFI

The calibration curve of AFM₁ GNP-LFI was constructed by plotting $1 - T/T_0$ against various concentrations of AFM₁. The data were then fitted by linear equations. Figure 7A shows the calibration curve exhibiting a linear range from 100 to $1,000 \text{ pg} \cdot \text{mL}^{-1}$ with a reliable coefficient of determination (R^2) of 0.9867. The regression equation could be represented by $y = 0.9040x - 1.7974$, where y is the inhibition rate and x is the logarithm of the AFM₁ concentration. Error bars were based on triplicate measurements at different AFM₁ concentrations. The LOD was defined as the mean minus the 3-fold standard deviation of the negative sample (Wu et al., 2017). The LOD of AFM₁ GNP-LFI was $50 \text{ pg} \cdot \text{mL}^{-1}$ and satisfied the European Union's maximum limit of AFM₁ in raw milk, heat-treated milk, and milk used to manufacture milk-based products and even in baby foods. Figure 7B presents the calibration curve constructed to detect *E. coli* O157:H7 by plotting the signal of T1 against *E. coli* O157:H7 concentration.

Figure 6. Signals of test (T) 1 line and T2 line with different concentrations of *Escherichia coli* O157:H7 and aflatoxin M₁ (AFM₁) in lateral flow immunoassay (LFI) setup I (—▲—) and setup II (—■—). Signals of the T line for the detection of *E. coli* O157:H7 (3.3×10^5 cfu·mL⁻¹) when AFM₁ was (A) 0 pg·mL⁻¹ or (B) 500 pg·mL⁻¹; for the detection of (C) AFM₁ (0 pg·mL⁻¹) or (D) AFM₁ (500 pg·mL⁻¹) when *E. coli* O157:H7 was 0 cfu·mL⁻¹; and for the detection of (E) AFM₁ (0 pg·mL⁻¹) or (F) AFM₁ (500 pg·mL⁻¹) when *E. coli* O157:H7 was 3.3×10^5 cfu·mL⁻¹. Competitive inhibition rates for the detection of AFM₁ (500 pg·mL⁻¹) when *E. coli* O157:H7 was (G) 0 cfu·mL⁻¹ or (H) 3.3×10^5 cfu·mL⁻¹. Error bars indicate SD (n = 3).

Figure 7. Standard calibration curve for aflatoxin M₁ (AFM₁) and *Escherichia coli* O157:H7. (A) Standard calibration curve for AFM₁ was obtained by plotting $1 - T/T_0$ against various AFM₁ concentrations. (B) Standard calibration curve for *E. coli* O157:H7 was obtained by plotting the signals of T line against various *E. coli* O157:H7 concentrations. (C) Strip figures of the standard curve (from 1 to 5, concentrations of AFM₁ were 0, 100, 200, 500, and 1,000 pg·mL⁻¹, respectively. Concentration of *E. coli* O157:H7 were 0, 3.3×10^3 , 3.3×10^4 , 3.3×10^5 , and 3.3×10^6 cfu·mL⁻¹, respectively). Data were based on 3 replicates. T and T₀ were the signals of the T line of negative and positive samples, respectively (Kong et al., 2016). Error bars indicate SD (n = 3). Color version available online.

The regression equation was $y = 431x - 1,750.7$, where y is the signal of T line and x is the logarithm of *E. coli* O157:H7 concentration with R^2 of 0.9608. The linear range was from 1.65×10^4 to 3.3×10^6 cfu·mL⁻¹. The LOD was defined as the mean plus 3-fold standard deviation of the negative sample (Rivas et al., 2015). The LOD for detecting *E. coli* O157:H7 in milk was 1.58×10^4 cfu·mL⁻¹.

Recovery Experiment

Table 1 lists the recoveries of GNP-LFI ranging from 78.0 to 111.6% with coefficients of variation in the range of 3.9 to 8.5% for the detection of AFM₁. For the detection of *E. coli* O157:H7, Table 2 shows that the range of recoveries was from 70.1 to 89.6% with CV ranging from 4.9 to 13.0%.

Detection of Real Sample

Ten raw milk samples were used to detect AFM₁ by using GNP-LFI test strips. As shown in Supplemental Table S1 (<https://doi.org/10.3168/jds.2018-14655>), AFM₁ was not detected in the raw samples by GNP-LFI

Table 1. Recovery experiment of gold nanoparticle-lateral flow immunoassay for the detection of aflatoxin M₁ (AFM₁)

Spiked AFM ₁ (pg·mL ⁻¹)	Mean ± SD	Recovery ¹ (%)	CV ² (%)
250	194.92 ± 7.55	78.0	3.9
450	375.33 ± 31.92	83.4	8.5
600	669.46 ± 54.44	111.6	8.1

¹Recovery = (detection concentration/spiked concentration) × 100%.

²CV = (SD/mean) × 100%.

test strips. The raw milk samples were then assayed by LC-MS/MS. Although AFM₁ was detected in sample 2 (3.2 pg/mL), sample 6 (5.1 pg/mL), and sample 9 (1.7 pg/mL), the concentrations of AFM₁ in the raw milk samples were lower than the LOD of the GNP-LFI test strips. *Escherichia coli* O157:H7 was not detected by GNP-LFI test strips and flat colony counting method in the raw milk samples (Supplemental Table S1; <https://doi.org/10.3168/jds.2018-14655>). These results showed that the GNP-LFI may be suitable for detection of real samples.

Specificity of GNP-LFI

The signals of GNP-LFI for detecting DON (10 ng/mL), OTA (10 ng/mL), and ZEN (10 ng/mL) were similar to those of the negative samples. No cross-reactivity was observed between AFM₁ and the mycotoxins (DON, OTA, and ZEN). Cross-reactivity was 88.3% between AFM₁ and AFB₁ (10 ng mL⁻¹) because AFM₁ is a hydroxylated metabolite of AFB₁ (panel A of Supplemental Figure S4; <https://doi.org/10.3168/jds.2018-14655>). Panel B of Supplemental Figure S4 shows the absence of cross-reactivity between *E. coli* O157:H7 (3.3×10^5 cfu·mL⁻¹) and 18 non-*Escherichia coli* O157:H7 (10^6 cfu·mL⁻¹). Slight cross-reactivity was found between *Staphylococcus aureus* and *E. coli* O157:H7 because protein A from *S. aureus* is well known to bind to the Fc region of most mammalian IgG classes (O'Seaghdha et al., 2006). These results obviously demonstrated the high specificity of GNP-LFI to simultaneously detect AFM₁ and *E. coli* O157:H7.

CONCLUSIONS

Aflatoxin M₁, which is a hydroxylated metabolite of AFB₁, has been classified as a group I carcinogenic agent. *Escherichia coli* O157:H7 is one of the most harmful foodborne pathogens that can cause serious illnesses, even death. Aflatoxin M₁ or *E. coli* O157:H7 may exist in raw milk. Aflatoxin M₁ is more suitable for detection with competitive assay format, whereas *E. coli* O157:H7 is more suitable for detection by sandwich assay. If AFM₁ or *E. coli* O157:H7 exists in milk, we can

use one test strip to detect any of them. Lateral flow immunoassay integrated with competitive and sandwich models was developed for the detection of AFM₁ and *E. coli* O157:H7. When competitive and sandwich models coexist in the LFI, the test line of sandwich model should be close to the sample pad to increase the signal of the positive sample, whereas the test line of the competitive model should be kept away from the sample pad to increase the inhibition rate. The LOD of LFI for detecting AFM₁ and *E. coli* O157:H7 were 50 pg·mL⁻¹ and 1.58×10^4 cfu·mL⁻¹, respectively, and showed good recovery and specificity.

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Table 2. Recovery experiment of gold nanoparticle-lateral flow immunoassay for the detection of *Escherichia coli* O157:H7

Spiked concentration (cfu·mL ⁻¹)	Mean (cfu·mL ⁻¹)	Recovery (%)	CV (%)
5.0×10^4	42,195	84.4	4.9
1.0×10^5	70,100	70.1	8.1
6.6×10^5	591,342	89.6	13.0

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