Development of an IgY-based lateral flow immunoassay for detection of fumonisin B in maize [version 1; peer review: 1 approved with reservations, 1 not approved]

Tien Viet Tran¹*, Binh Nhu Do (ID)¹*, Thao Phuong Thi Nguyen², Tung Thanh Tran (ID)², Son Cao Tran³, Ba Van Nguyen¹, Chuyen Van Nguyen¹, Hoa Quang Le²

¹Vietnam Military Medical University, Hanoi, 100000, Vietnam
²School of Biotechnology and Food Technology, Hanoi University of Science and Technology, Hanoi, 100000, Vietnam
³Laboratory of Food Toxicology and Allergens Testing, National Institute for Food Control, Hanoi, Vietnam

* Equal contributors

Abstract
Fumonisin is one of the most prevalent mycotoxins in maize, causing substantial economic losses and potential health risks in human and animals. In the present study, in-house polyclonal IgY antibody against fumonisin group B (FB) was applied for the development of a competitive lateral flow immunoassay detecting these mycotoxins in maize grains with the limit of detection of 4000 µg/kg, which corresponds to the maximum residue limit adopted by The International Codex Alimentarius Commission. To this end, factors affecting the test performance including nitrocellulose membrane type, dilution factor of maize homogenates in running buffer, amount of detection conjugate, and incubation time between detection conjugate and samples were optimized. Under the optimal condition (UniSart® CN140 nitrocellulose membrane, FB₁-BSA immobilized at 1 µg/cm², 1:10 dilution factor, 436 ng of gold nanoparticle conjugate, 30 minutes of incubation), the developed test could detect both FB₁ and FB₂ in maize with limit of detection of 4000 µg/kg, and showed no cross-reactivity to deoxynivalenol, ochratoxin A, aflatoxin B1 and zearalenone. When applied to detect FB₁ and FB₂ in naturally contaminated maize samples, results obtained from the developed assay were in good agreement with those from the high-performance liquid chromatography method. This lateral flow immunoassay is particularly suitable for screening of fumonisins in maize because of its simplicity and cost-effectiveness.

Keywords
fumonisin B, rapid methods, lateral flow immunoassay, IgY
Corresponding author: Hoa Quang Le (hoa.lequang@hust.edu.vn)

Author roles: Tran TV: Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Project Administration, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing; Do BN: Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Project Administration, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing; Nguyen TPT: Data Curation, Formal Analysis, Investigation, Methodology, Validation, Writing – Original Draft Preparation; Tran TT: Data Curation, Formal Analysis, Investigation, Validation, Writing – Original Draft Preparation, Writing – Review & Editing; Tran SC: Investigation, Methodology, Writing – Review & Editing; Nguyen BV: Formal Analysis, Funding Acquisition, Investigation, Project Administration, Resources, Validation, Writing – Original Draft Preparation; Nguyen CV: Formal Analysis, Funding Acquisition, Investigation, Project Administration, Resources, Validation, Writing – Original Draft Preparation; Le HQ: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Project Administration, Supervision, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

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**Introduction**

Fumonisins are a group of mycotoxins from *Fusarium* species, mostly *Fusarium proliferatum* and *Fusarium verticillioides* (Gelderblom et al., 1988). In terms of chemical and physical characteristics, fumonisins are soluble in water and methanol; and are heat-stable over a wide range of processing unless the temperature exceeds 150°C (Jackson et al., 1996; NTP, 2001; Yazar & Omurtog, 2008). To date, four groups of fumonisin have been identified (A, B, C and P-series) (Rheeder et al., 2002), among which fumonisin B (FB) is the most common mycotoxins found in corn, and have been shown to have various toxic and carcinogenic effects (Munkvold et al., 2019; NTP, 2001). Due to its potential toxicity, the International Codex Alimentarius Commission has adopted the maximum level for the presence of fumonisins in raw maize at 4000 µg/kg (EC, 2005).

Several recent studies pointed out fumonisin contamination in corn represents a major public-health concern in diverse countries including China (Fu et al., 2015; Guo et al., 2016; Hu et al., 2019; Liu et al., 2017), Brazil (Scussel et al., 2014), Kenya (Mutiga et al., 2015), South Africa (Mngqawa et al., 2016), Malawi (Mwalwayo & Thole, 2016), Tanzania (Kamala et al., 2016), Nigeria (Chilaka et al., 2016), Ethiopia (Getachew et al., 2018), Somalia (Wielogorska et al., 2019). In Vietnam, Hieu Phuong et al. (2015) showed that FBs were the major mycotoxin that contaminated maize with 67% of incidence, a range of positive samples for FB1 and FB2 at 102 to 10799 µg/kg and 102–5051 µg/kg respectively.

Conventionally, FBs could be detected by chromatography and immunological methods. To date, high performance liquid chromatography (HPLC), liquid chromatography tandem mass spectrometry (LC-MS/MS) and gas chromatography-mass spectrometry (GC-MS) methods have been developed for FBs analysis. However, they are laborious, time consuming and require specialized equipment. On the other hand, lateral flow immunosassays (LFIA) are cost-effective, easy to use and suitable for on-site analysis. Several LFIs have been developed for sensitive detection of FBs using monoclonal antibodies (Wang et al., 2013; Wang et al., 2014; Yu et al., 2015) or rabbit polyclonal antibodies (Anfossi et al., 2010; Venkataramana et al., 2014).

Polyclonal IgY antibodies from egg yolk of laying hens represent an attractive alternative to monoclonal and rodent polyclonal antibodies. With one course of immunization, IgY could be extracted non-invasively in a large quantity (up to 40–80 mg), with 2–10% of which being antigen specific (Kovacs-Nolan & Mine, 2004; Pauly et al., 2011). As a result, IgY has been increasingly employed for the development of cost-effective rapid tests. Its usefulness in LFIs has been demonstrated for detection of morphine (Gandhi et al., 2005), methicillin-resistant *Staphylococcus aureus* (Yamada et al., 2013), staphylococcal enterotoxins (Jin et al., 2013), and rhein (Zhang et al., 2018).

In the present study, we demonstrated the development of a gold nanoparticle-based LFIA that used in-house polyclonal IgY for simple and cost-effective screening of FBs in maize.

**Methods**

**Preparation of FB1-BSA conjugate**

Conjugation of FB1 to BSA was performed following the protocol by Szurdoki et al. (1996) with some modifications. Glutaraldehyde (GA) solution 50 % (W/V) (Sigma-Aldrich, Cat Nº 340855) was used as the cross-linker reagent while BSA (Sigma-Aldrich, Cat Nº A9085) was used as the carrier protein. Specifically, BSA (5 mg/mL) was dialyzed in 20 mM sodium phosphate buffer pH 6.0. A total of 10 µl of GA 50% (W/V) were then incubated with 1 mL of the dialyzed BSA solution overnight at room temperature. After incubation, excess GA was removed by dialyzing in Phosphate-buffered saline (PBS), followed by addition of 1 mg of FB1 (Santa Cruz Biotechnology, Cat Nº sc-201395A) to achieve the molar ratio of 20:1 (FB1:BSA-GA). The mixture was incubated at 4°C overnight on a Dynal Biotech rotary shaker (10 rpm) before the addition of 80 µl of glycine 1 M (Bio Basic, Cat Nº GB0235) to block unreacted aldehyde groups. The reaction mixture was further incubated at room temperature for 4 hours. Subsequently, sodium borohydride powder (Sigma-Aldrich, Cat Nº 452882) was added to the mixture (final concentration of 10 mg/mL) and incubated for 4 hours at room temperature. The obtained solution was then dialyzed and concentrated in 10 mM Borat buffer pH 8.5 using a 10 kDa Amicon® Ultra-4 Centrifugal Filter Unit (Millipore, Cat Nº UFC801024). Lastly, FB1-BSA conjugate was quantified using Nano Drop 2000 (Thermo Fisher Scientific) and stored at 4 °C.

**Production of IgY against FB1-KLH**

**Animal procedures.** All animal procedures were approved by the Research Ethics Committee of Vietnam Military Medical University. All efforts were made to ameliorate harm to the animals, by conforming to the Principles of animal care and use in research adopted by the Vietnam Military Medical University.

A total of two Fayouni hens (aged 20 weeks) were sourced from Thuy Phuong Poultry Research Center, Vietnam for IgY production. Hens were housed individually in standard battery cages (800 cm²/hen) and received commercial rations (A55, Anova Feed) and water *ad libitum*. The temperature was kept between 25 and 35°C and the light cycle was 16 hours light/8 hours dark.

Polyclonal IgY antibody against FB1-KLH was obtained as described previously (Do et al., 2016). Briefly, FB1-KLH was prepared according to procedures described by Szurdoki et al. (1996). Glutaraldehyde (GA) solution 50 % (W/V) (Sigma-Aldrich, Cat Nº 340855) was used as the cross-linker reagent. A total of 10 mg of KLH (Thermo Fisher Scientific, Cat Nº 77600) was dissolved in 12 mL of water and dialyzed against 2 L of 0.2% GA in 0.01 M PBS (pH 7.5) for 20 hours. Excess GA was removed by dialyzing in PBS, followed by dropwise addition of 2 mg of FB1 (Santa Cruz Biotechnology, Cat Nº sc-201395A). The mixture was incubated at 4°C overnight on a Dynal Biotech rotary shaker (10 rpm) before the addition of 80 µl of glycine 1 M (Bio Basic, Cat Nº GB0235) to block unreacted aldehyde groups. The reaction mixture was further incubated at room temperature for 4 hours. The obtained
solution was then dialyzed and concentrated in PBS pH 7.5 using a 100 kDa Amicon® Ultra-4 Centrifugal Filter Unit (Millipore, Cat No. UFC810004). Lastly, FB1-KLH conjugate was quantified using Nano Drop 2000 (Thermo Fisher Scientific) and stored at 4°C.

The chickens were intramuscularly immunized three times in 10 days intervals to elicit strong immune response. For the first immunization, an injection dose of 1.0 mL was prepared by mixing 0.2 mg of FB3-KLH with an equal volume of complete Freund’s adjuvant (Sigma-Aldrich, Cat No. F5881). For the two subsequent booster immunizations, the amount of immunogen was decreased to 0.1 mg of FB3-KLH and incomplete Freund’s adjuvant (Sigma-Aldrich, Cat No. F5506) was used. Eggs were collected two weeks after the last immunization and stored at 4°C. The extraction of IgY was performed by polyethylene glycol (PEG) (Sigma-Aldrich, Cat No. 81255) precipitation as described by Pauly et al. (2011). The eggshell was carefully cracked, and the yolk was transferred to a “yolk spoon” and filter paper to remove egg white. The egg yolk skin membrane was cut before the yolk was poured into a 50 ml tube. Twice the egg yolk volume of PBS was added to the tube and mixed by vortexing. PEG 6000 was added to achieve the final concentration of 3.5% (w/v) and the tube was vortexed and rolled for 10 minutes on a Dynal Biotech rotary shaker (30 rpm) before being centrifuged at 8000 × g, 4°C for 10 minutes. The supernatant was subjected to filtration and then to precipitation of IgY by adding PEG 6000 (final concentration 12% (w/v)). The tube was vortexed and centrifuged at 8000 × g, 4°C for 30 minutes and the supernatant was discarded. The pellet was dissolved in 10 mL PBS and PEG 6000 was added to achieve the final concentration of 12% (w/v). Subsequently, the tube was centrifuged at 8000 × g, 4°C for 30 minutes. The pellet was dissolved in 5 mL of PBS and IgY was further purified by microfiltration via a 0.45 µm membrane and ultrafiltration using 100 kDa Amicon® Ultra-4 Centrifugal Filter Units (Millipore, Cat No. UFC810008). Finally, IgY was stored at -80°C in small aliquots.

Preparation of IgY-conjugated gold nanoparticles

IgY-gold nanoparticle conjugate was prepared using BioReady 40 nm Carboxyl Gold (Nanocomposix, Cat No. AUXR40-5M). Particularly, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Sigma-Aldrich, Cat No 03449) and N-hydroxysulfosuccinimide (Sulfo-NHS) (Sigma Aldrich, Cat No 56485) at 10 mg/mL in H2O were freshly prepared before conjugations. Anti-FB IgY was dialyzed in Antibody purification buffer (10 mM potassium phosphate, pH 7.4) using Amicon Ultra-0.5 Centrifugal Filter Unit (Millipore, Cat No. UFC501096). One milliliter (0.83 mg) of BioReady 40 nm Carboxyl Gold was mixed with 20 µl and 40 µl of the prepared EDC and Sulfo-NHS respectively. The mixture was then incubated on a Dynal Biotech rotary shaker (15 rpm) at room temperature for 30 minutes then centrifuged at 3600 × g for 10 minutes. The supernatant was then removed completely, and the gold nanoparticles were resuspended in 1 mL of Reaction Buffer (5 mM potassium phosphate, 0.5% 20K MW PEG, pH 7.4). The mixture was then incubated with 50 µg of anti-FB IgY on a Dynal Biotech rotary shaker (15 rpm) at room temperature for 2 hours. Subsequently, blocking of remaining NHS-esters was performed using 10 µl of 50% (w/v) hydroxylamine. IgY-conjugated gold nanoparticles were then washed three times with 1 mL of Reaction Buffer. Lastly, gold nanoparticle conjugate was resuspended in 10 mL of Conjugate Diluent (0.1X PBS, 0.5% BSA, 0.05% Sodium Azide) and stored at 4°C.

Preparation of LFIA test strips

Test strips were prepared following Posthuma-Trumpie et al. (2008) with some modifications. Briefly, a Linomat V (Camag, Cat No. 022.7808) was used to dispense FB3-BSA and Mouse monoclonal 0.8C Anti-Chicken IgY H&L (Abcam, Cat No. ab82229) at the test line and control line positions of a nitrocellulose membrane respectively. For the control line, immunoglobulins were dispensed at a dose of 0.5 µg/cm, at the position of 2 cm away from the dipping point. For the test line, FB3-BSA was dispensed at a dose of 1 µg/cm at the position of 1.5 cm away from the dipping point. The membrane was then dried for 2 hours at 37°C. A second plastic backing and an absorption pad (Extra Thick Blot Paper, BIO-RAD, Cat No. 1703969) were applied; and the membranes were cut into 4 mm-wide test strips using an Autokun cutter (Hangzhou Autokun Technology). Test trips were sealed in aluminum packages with a desiccation pad and stored at 4°C until use. Three different membranes were tested, namely CNPC-3S12, 10 µm (MDI technologies, Cat No. CNPC-SS12-10µm-25mm), UniSart®, CN140 (Sartorius, Cat No. 1UN4ER100025NTB), and UniSart® CN 95 (Sartorius, Cat No. 1UN95ER100040WS).

Sample preparation and assay procedures

Blank and naturally contaminated maize grains were collected from local markets in Hanoi, Vietnam during the year of 2017. The samples were finely ground using an A 11 basic Analytical mill (IKA) and a 500 µm sieve.

Spiking of FBs into maize was performed on a blank sample. Briefly, 5 g of ground maize were spiked with 10–40 µl of FB or FB stock solution of 1 mg/mL to achieve final content of 2000 – 8000 µg/kg. Spiked samples were left 24 hours at 4°C. Extraction of FBs and LFIA analysis were performed as described below.

The protocol for FB extraction from naturally contaminated or spiked samples (Figure 1) was based on the work of Pietri & Bertuzzi (2011) and Lattanzio et al. (2012). Instead of using organic solvents, FB was extracted with 0.4 M phosphate buffer (PB) at pH 7.5 (Pietri & Bertuzzi, 2011). Specifically, 5 g of maize flour were mixed with 45 mL of PB and blended using a T10 basic ULTRA-TURRAX® (IKA) at the highest speed for 3 minutes. The blended samples were then allowed to settle for 3 minutes to recover the supernatant, which was further diluted 1:3, 1:5, 1:10 or 1:20 in Running Buffer (100 mM Borat Buffer, 0.5% BSA, 0.05% Tween®-20, 0.02% NaNO3, pH 8.5). For LFIA analysis, 100 µl of the diluted extracts were dispensed into a 2-mL lyophilization glass vials and incubated with 174 ng, 436 ng or 697 ng (corresponding to 2, 5, 8 µl) of detection conjugate for 0 to 60 minutes before
Figure 1. Schematic representation of fumonisin B extraction and lateral flow analysis for maize samples. A total of 5 g of maize were homogenized in 45 mL of phosphate buffer for 3 minutes. The mixture was allowed to settle for 3 minutes before collection of the supernatant, which was further diluted in running buffer. A hundred microliters of the diluted extract were used for lateral flow immunoassays (LFIA) analysis. After being incubated with detection conjugate at room temperature, samples were flowed onto LFIA strips. Results were read with the naked eye after the strips absorbed fluid completely.
recognition specificity toward both FB1 and FB2 (Do et al., 2016), is conjugated to gold nanoparticle (see underlying data (Tran et al., 2019)). The labeled antibody was mixed with the sample extract in a glass vial, and the mixture was incubated to allow antigen-antibody complexes to form before flowing onto the nitrocellulose membrane which contains a test line and a control line. In our assay, FB1-BSA conjugate was immobilized on the test line while a secondary antibody against chicken IgY was coated on the control line. In a negative sample, the free detection antibody binds to the FB1-BSA conjugate immobilized on the test line, forming a visible line. An excess of the labeled antibody migrates to the control line and binds to the secondary antibody. As a result, a negative sample will form two visible lines on the nitrocellulose membrane. In a positive sample, FBs in the sample extract will react with all of the available binding sites of the antibody, thus preventing attachment of the detection antibody to the FB1-BSA conjugate on the test line. All of the detection conjugate will migrate to the control line and will form a visible line. Consequently, a positive sample will form only one line at the control zone.

Optimization has been performed with FB1, the most common mycotoxin in maize, so that the samples with FB1 concentration equal to or beyond the maximum residue limit of 4000 µg/kg, will result in no visible line at the test zone. To this end, the effects of nitrocellulose membrane type, dilution factor of maize homogenates in running buffer, amount of detection conjugate, and the incubation time between sample extract and detection conjugate, on the test performance were evaluated.

Selection of nitrocellulose membrane. Flow rate and protein-binding capacity of nitrocellulose membranes directly affect sensitivity and run time of a LFIA (O’Farrell, 2008). Generally, nitrocellulose membranes with a low flow rate will facilitate the formation of immunocomplexes at the test and control lines. However, it could lead to extended run times and false positive results (O’Farrell, 2008). In the present study, selection of nitrocellulose membrane was carried out by analyzing running buffer mixed with detection conjugate (negative controls) on three different nitrocellulose membranes. Figure 2 indicated that UniSart® CN140 (Sartorius) and CNPC-SS12 10 µm (MDI technologies) produced higher signal intensities than UniSart® CN95 (Sartorius). Although the difference in signal intensity between UniSart® CN140 and CNPC-SS12, 10 µm was not statistically significant (p = 0.9209), sample uptake time was significantly lower on UniSart® CN140 (Figure 2B). Therefore, UniSart® CN140 from Sartorius was chosen for subsequent experiments.

Optimization of dilution factor of maize extract. Food sample extracts are commonly diluted before analysis by LFIA to minimize the negative effects of sample matrix on antibody-antigen reactions (Anfossi et al., 2011; Lattanzio et al., 2012). To determine the optimal dilution factor, blank maize grains were subjected to extraction using phosphate buffer (PB).
and dilution in running buffer with ratios ranging from 1:3 to 1:20. According to Pietri & Bertuzzi (2011), average recovery percentages were 95.5±1.9% and 96.7±2.1% for FB₁ and FB₂ when extracted in PB. Furthermore, this extraction method does not require the use of toxic solvents and it may prevent possible inhibiting effects of organic solvents on antibody-antigen reaction (Rehan & Younus, 2006; Russell et al., 1989).

Test line intensities generated by the diluted extract samples were compared with those from negative controls. Figure 3 revealed that signal intensities were decreased at dilution factors of 1:3 and 1:5, comparing to those from negative controls. However, starting from 1:10 dilution, test line signals were similar to negative controls (Figure 3B). As a result, an optimal dilution factor of 1:10 was set for subsequent experiments.

Optimization of amount of detection conjugate. Quantity of labeled antibody directly affects the limit of detection of a competitive LFIA. In fact, if a low amount of detection conjugate is used, no visible test lines will be formed even low levels of FBs (less than 4000 µg/kg) are present in the samples. Furthermore, using a low amount of detection conjugate will decrease the signal intensities at both test and control lines, causing difficulties in result interpretation. On the other hand, using an excessive amount of detection conjugate will negatively affect the analytical sensitivity of the assay as more toxins are required to saturate all the binding sites of the detection antibody.

In the present study, various amounts (174 ng, 436 ng, and 697 ng corresponding to 2, 5, and 8 µl) of detection conjugate were used to react with FB₁ extracted from blank samples spiked with this toxin at 2000 µg/kg, 4000 µg/kg or 8000 µg/kg. For samples spiked with 2000 µg/kg FB₁, test lines were observed on all test strips regardless of the amounts of detection conjugate used (Figure 4). Conversely, no test line was observed when FB₁ is present at 8000 µg/kg (Figure 4). At the cut-off level of 4000 µg/kg, test line signal was still present when 697 ng of detection conjugate were used while no test line was visible when 174 ng or 436 ng of detection conjugate were used. However, using 174 ng of detection conjugate resulted in low signals of test line on negative controls (Figure 4). Therefore, 436 ng of IgY-conjugated gold nanoparticles were used for further studies.

Optimization of incubation time between samples and detection conjugate. Effects of incubation step between detection conjugate and FB₁ at the cut-off level (extracted from blank

![Figure 3. Optimization of dilution factor of maize extract.](image-url)
Figure 4. Optimization of amount of detection conjugate to achieve the detection limit of 4000 µg/kg of fumonisin B₁ (FB₁). (A) Images of negative controls and diluted extracts of blank maize samples spiked with FB₁ at 2000 µg/kg, 4000 µg/kg, and 8000 µg/kg using 174 ng, 436 ng or 697 ng of detection conjugate on representative lateral flow immunoassay (LFIA) strips. Maize extracts were incubated with detection conjugate for one hour at room temperature before being analyzed on LFIA strips. Experiment was performed with 8 replicates. CL, control line; TL, test line. (B) Quantification of test line signals. AU, arbitrary unit.

samples spiked with FB₁ at 4000 µg/kg) on the test performance were assessed by varying the incubation time from 0 to 60 minutes. Results (Figure 5) indicated that test lines were still visible when the incubation time was 0 or 15 minutes, while no test lines were observed when the incubation time was 30 or 60 minutes. To shorten the analytical procedure, an incubation time of 30 minutes was chosen.

Determination of limit of detection of the developed LFIA for FB₂ in maize

Previously, we have shown that the polyclonal IgY antibody used in the present study, recognized FB₁ and FB₂ with different affinities (IC₅₀ = 10 and 49 ng/ml for FB₁ and FB₂ respectively) (Do et al., 2016). To determine if the developed LFIA could detect FB₂ in maize at the cut-off level of 4000 µg/kg, this toxin was spiked into a blank sample at 2000 µg/kg, 4000 µg/kg, and 8000 µg/kg. Results (Figure 6) showed that no visible line was formed at test zone for samples spiked with 4000 µg/kg and 8000 µg/kg of FB₂. On the contrary, faint signals were still observed at the test line for samples spiked with 2000 µg/kg of FB₂. Therefore, the limit of detection of our LFIA for FB₂ was also 4000 µg/kg, meaning that the developed test could be used for screening of total FBs in maize. The extended incubation time between detection conjugate and the toxins (30 minutes) and the optimal amount of labeled antibody are likely able to compensate for the difference in affinity of IgY antibody for FB₁ and FB₂.

Cross-reactivity tests

Cross-reactivity tests were performed using deoxynivalenol, ochratoxin A, aflatoxin B1 and zearalenone spiked into blank maize extracts at two different concentration levels (100-fold and 1000-fold of MRL). Figure 7 showed that there was no difference in signal intensities at test line position between negative control and samples spiked with low and high concentrations of the tested mycotoxins. Therefore, the developed LFIA did not cross-react with deoxynivalenol, ochratoxin A, aflatoxin B1 and zearalenone.

Analysis of naturally contaminated maize samples by IgY LFIA

A total of 19 maize samples were analyzed by HPLC-MS and the developed assay. By HPLC-MS, all samples were negative with FB₂, while 11 samples were positive with FB₁, with
concentrations ranging from 27 µg/kg to 7850 µg/kg (Table 1). One sample (No 16: 7850 µg/kg of FB₁) exceeded the maximum legislative limits (EU) of 4000 µg/kg (CEN, 2001). Notably, only this sample was positive by IgY LFIA. Although the sample size was relatively low, these findings indicated that results by the IgY LFIA were in good agreement with those from HPLC-MS method as no false positive or false negative results were found.

**Conclusions**

In conclusion, this is the first study using polyclonal IgY antibody in LFIA for simple detection of FBs in maize with the

**Figure 5.** Optimization of incubation time between detection conjugate and samples to be analyzed. (A) Images of diluted extract (from blank sample spiked with fumonisin B₁ (FB₁) at 4000 µg/kg) incubated with 436 ng of detection conjugate for 0, 15, 30 and 60 minutes on representative lateral flow immunoassay strips. 1, negative control; 2, 3, 4, 5, incubation time of 0, 15, 30, 60 minutes respectively; CL, control line; TL, test line. (B) Quantification of signal intensities. NC, negative control; AU, arbitrary unit.

**Figure 6.** Limit of detection of fumonisin B₂ (FB₂) in maize by the developed lateral flow immunoassay. (A) Images of negative controls (1) and diluted extracts of blank maize sample spiked with FB₂ at 2000 µg/kg (2), 4000 µg/kg (3), and 8000 µg/kg (4) on representative lateral flow immunoassay strips. CL, control line; TL, test line. Experiment was performed with 8 replicates. (B) Quantification of test line signals. AU, arbitrary unit.

**Figure 7.** Cross-reactivity of the developed lateral flow immunoassay against deoxynivalenol, ochratoxin A, aflatoxin B₁, and zearalenone. Deoxynivalenol (1750 and 17500 ng/mL), ochratoxin A (5 and 50 ng/mL), aflatoxin B₁ (10 and 100 ng/mL), zearalenone (350 and 3500 ng/mL) were spiked into diluted extracts of blank maize samples and incubated with detection conjugate for 30 minutes before being analyzed on lateral flow immunoassay (LFIA) test strips. Negative, negative controls; Positive, 40 ng/mL fumonisin B₁ (FB₁), CL, control line; TL, test line.
Table 1. Analysis of naturally contaminated maize samples by high performance liquid chromatography–mass spectrometry (HPLC-MS) method and the IgY Lateral Flow Immunoassay.

<table>
<thead>
<tr>
<th>Sample</th>
<th>FB1 concentration by HPLC-MS (µg/kg)</th>
<th>FB2 concentration by HPLC-MS (µg/kg)</th>
<th>Analysis by the developed LFIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1760</td>
<td>Not detected</td>
<td>Negative</td>
</tr>
<tr>
<td>2</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Negative</td>
</tr>
<tr>
<td>3</td>
<td>183</td>
<td>Not detected</td>
<td>Negative</td>
</tr>
<tr>
<td>4</td>
<td>95</td>
<td>Not detected</td>
<td>Negative</td>
</tr>
<tr>
<td>5</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Negative</td>
</tr>
<tr>
<td>6</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Negative</td>
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<tr>
<td>7</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Negative</td>
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<tr>
<td>19</td>
<td>322</td>
<td>Not detected</td>
<td>Negative</td>
</tr>
</tbody>
</table>

limit of detection meeting the EU regulatory requirements for MRL of 4000 µg/kg. The main advantage of this assay is its cost-effectiveness as one single egg from inoculated hens yielded approximately 8 mg of purified IgY, which is enough to produce 320000 tests. One limitation of the developed LFIA is that the analysis times are longer than those of commercial assays as it requires an incubation step of 30 minutes to allow FBs to saturate the binding sites of detection antibody. However, as mentioned above, this additional step was likely able to compensate for the difference in affinity of IgY antibody for FB1 and FB2, enabling the new assay to meet the cut-off level of 4000 µg/kg for both these toxins.

Data availability

Underlying data

Figshare: Raw data for “Development of an IgY-based lateral flow immunoassay for detection of fumonisin B in maize”. https://doi.org/10.6084/m9.figshare.8320775 (Tran et al., 2019)

This project contains the following underlying data:

- Dataset 1_Raw scans of lateral flow test strips.rar (Folder includes raw images of lateral flow strips)
- Dataset 2.pptx (PowerPoint file includes HPLC-MS chromatograms of naturally contaminated samples for quantification of FB1 and FB2.)
- Dataset 3.xlsx (Excel file includes quantification of signal intensities of lateral flow test strips and quantification of FB1 and FB2 from HPLC-MS output.)

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

Grant information

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

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Reference Source


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Version 1

Reviewer Report 23 August 2019

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Zhanhui Wang
Beijing Advanced Innovation Center for Food Nutrition and Human Health, Beijing Key Laboratory of Detection Technology for Animal-Derived Food Safety, China Agricultural University, Beijing, China

The paper described an IgY-based lateral flow immunoassay for detection of fumonisin B in maize. In general, the paper is more like an experimental report than scientific study in my opinion. The paper provided little new information for readers and the commercial products of LFIA for FBs are already available in the market with high performances. All techniques used in the study are conventional and no any improvement was achieved.

1. The LFIA for the rapid detection of FBs are reported by using many probes not only gold. The performance of the LFIA developed by the authors in term of sensitivity, specificity and accuracy are not comparable with those of reports.

2. The novelty of the study relied on the first report of usage of IgY in LFIA for FB in my opinion, however, the production of IgY to FB already reported by the authors. The paper should show the advantages and disadvantages of usage of IgY in LFIA. And there are no comparative data with other antibodies like antibody from mouse or rabbit. Exactly, the production of IgY to FB is also a well established techniques.

3. The main body of the manuscript is the optimization of LFIA conditions, the procedure of optimization is necessary for any analytical methods and not the point of the study.

4. I do not think the IgY used in the study is the first choice since the affinity and specificity of IgY is inferior in comparison with reported antibodies, which may result in inaccuracy determination.

5. The authors should provide the confirmation data for protein conjugates, gold conjugates if they are firstly reported or just cited the reference if these data already reported. For LFIA, the gold-antibody is important, why the authors have not optimized the preparation of gold-protein conjugates? The low sensitivity of the LFIA maybe derived from the coating antigens and suggest the authors should evaluate different coating antigens differentiating with ratio, hapten and conjugation methods. In addition, the authors should test the cross-reactivity with FB3 since the analog is available and a potential interferent.
In conclusion, the paper did not describe a new experimental, observational, or computational method, test or procedure. I have to be against indexing and hope the suggestions could help the authors improve the study.

Is the rationale for developing the new method (or application) clearly explained?
Partly

Is the description of the method technically sound?
Partly

Are sufficient details provided to allow replication of the method development and its use by others?
Partly

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Analytical Chemistry, Food Safety, Antibody production and immunoassay.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.
for example among the fumonisin FB1 is more potent and frequently reported one in may kind of food grains mostly in wheat and maize and the cross reactivity patterns with group of Fusarium toxins need to be studied for example DON, T-2 toxin, Nivalenol etc.

- Introduction part is too vague, need to be improved with recent and relevant references.
- Preparation of IgY-conjugated gold nano particles is not clear, it's totally confusing, authors should clear draft the protocol in a supplementary section or main text.
- Ethical committee guidelines and permission to carry out the study need be given the manuscript.
- **Selection of nitrocellulose membrane: its very crucial in developing the LFA assay, in the present study sufficient information was not presented by the authors in this section. please be give the clear protocol how its been chosen.**
- OD 4000ug/kg is very high, many studies reported that the LD is under nano gram levels, this need to be further improved for their sensitivity.
- Over study looks good and can be indexed in this journal after substantial revision of the manuscript.

Is the rationale for developing the new method (or application) clearly explained?
Partly

Is the description of the method technically sound?
Partly

Are sufficient details provided to allow replication of the method development and its use by others?
Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Toxicology and Immunology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.
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