EVALUATION OF A COMPETITIVE IMMUNOASSAY DIPSTICK TEST FOR THE DETECTION OF AFLATOXIN B1 AT LABORATORY SCALE

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Received: 10/06/2025
Revised: 17/06/2025; Accepted: 21/06/2025

ABSTRACT

Objective: To determine key technical parameters of a competitive immunoassay dipstick for the rapid detection of Aflatoxin B1 (AFB1).

Methods: Optimization was carried out for several parameters, including the concentration of coating antigen on the test line, storage conditions of the antibody–gold nanoparticle conjugate, the composition of the running buffer, and the volumes of reagents applied during testing. Results: The developed test strip demonstrated a detection limit of 0.01 µg/mL, with no cross-reactivity observed against standard samples of Aflatoxin M1, Ochratoxin A, and Patulin at tested concentrations. The test exhibited a sensitivity of 87.5% and a specificity of 95%.

Conclusion: A competitive immunoassay dipstick test for the rapid detection of AFB1 was successfully developed by optimizing the antibody–gold conjugation process, the running buffer composition, and strip configuration. The test shows strong potential for on-site AFB1 screening in food safety monitoring.

Keywords: Competitive immunoassay dipstick; Rapid detection; Aflatoxin B1; Sensitivity.

1. INTRODUCTION

Aflatoxin B1 (AFB1) is a mycotoxin belonging to the aflatoxin group, produced primarily by Aspergillus flavus and Aspergillus parasiticus under tropical and subtropical conditions [1]. It is among the most prevalent contaminants found in agricultural products and foodstuffs, including maize, peanuts, soybeans, and other cereals. The International Agency for Research on Cancer (IARC) has classified AFB1 as a Group 1 carcinogen-carcinogenic to humans [2]. Notably, AFB1 is recognized for its hepatotoxicity, immunosuppressive effects, and genotoxic potential, posing significant health risks to both humans and animals [3][4].

To monitor AFB1 contamination levels in food, highly accurate analytical methods such as high-performance liquid chromatography (HPLC), gas chromatography (GC), and mass spectrometry (MS) are commonly employed [5]. However, these methods require expensive instrumentation, complex procedures, and extended analysis time, rendering them unsuitable for on-site applications or in settings with limited infrastructure, such as remote areas, small-scale facilities, or field-deployed military units.

In recent years, rapid testing techniques, particularly lateral flow immunoassays (LFIA), have garnered attention due to their simplicity, quick turnaround, and minimal equipment requirements [6][7]. LFIAs commonly use gold nanoparticles as color indicators, allowing for visual interpretation of results within 5–15 minutes [8]. These characteristics make LFIA an ideal tool for rapid, field-based screening of AFB1 without requiring advanced technical expertise or extensive sample preparation.

Based on these practical needs, we conducted a study to evaluate the performance of a competitive immunochromatographic dipstick assay for the rapid detection of AFB1 under laboratory conditions.

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2. MATERIALS AND METHODS

2.1. Reagents and Materials

- Detection Antibody: Monoclonal anti-AFB1 antibody (OAEF00325) purchased from Biotrend.

- Test Line Antigen: AFB1-BSA conjugate (32754) obtained from Sigma; this antigen is immobilized on the test line in the competitive format.

- Control Line Antibody: Anti-mouse IgG (M5899) from Sigma.

- Standard Mycotoxins: AFB1, Aflatoxin M1, Ochratoxin A, Patulin antigen

- Chemicals: Borate buffer, TBS, PBS, Tween 20, NaCl, sucrose, and lactose were purchased from Sigma.

- Gold Nanoparticles: 30 nm nanoparticles synthesized at the Military Hygiene Department Laboratory, Vietnam Military Medical University.

- Membranes: Nitrocellulose membrane (FF90HP, Whatman), upper absorbent pad (CF4, Whatman), and backing card.

2.2. Conjugation of Anti-AFB1 Antibody with Gold Nanoparticles

Gold nanoparticles (~30 nm) synthesized in-house were functionalized with the monoclonal anti-AFB1 antibody through passive adsorption. The pH of the colloidal gold was adjusted to match the isoelectric point (pI) of the antibody. The optimal conditions for conjugation included: 50 µg/ml antibody, borate buffer at pH 7.2, reaction temperature of 32°C, and a gold colloid solution with OD540 = 25 in a 0.7 ml volume.

Post-conjugation, the gold–antibody complex was blocked using 1% BSA, 5% sucrose, and 1% Tween 20 in borate buffer (pH 9). The mixture was gently agitated for 16 hours at room temperature to block nonspecific binding sites. The final conjugates were stored at 4°C and characterized using Zeta potential measurements and Dot-Blot analysis.

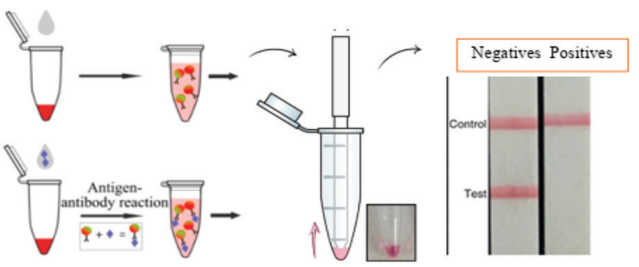


Figure 1. Schematic representation of AFB1 detection using the competitive LFIA dipstick

2.3. Dipstick Fabrication

Nitrocellulose membrane (FF90HP) was treated with borate buffer (pH 7.2) containing 2% BSA, 1% Tween 20, and 0.2% sucrose to enhance membrane performance. The test line was immobilized with 1 mg/ml AFB1-BSA, and the control line was coated with 1 mg/ml anti-mouse IgG.

To maintain the stability of the gold–antibody complex, a storage buffer consisting of borate buffer (pH 7.4), 1% BSA, 1% Tween 20, and 3% sucrose was optimized. The running buffer was Tris buffer (70 mM, pH 6.8) containing 0.5% Tween 20, 0.5% glycine, and 0.5% sucrose.

The optimized assay protocol involved using 24 µl of running buffer and 1 µl of gold–antibody complex per test. Components were assembled accordingly to finalize the immunochromatographic strip.

2.4. Cross-Reactivity Evaluation

Cross-reactivity was evaluated using standard mycotoxins: Aflatoxin M1 (AFM1), Ochratoxin A (OTA), and Patulin at concentrations ranging from 1 to 50 ng/ml. The relative inhibition effects were compared to those produced by specific concentrations of AFB1 to assess nonspecific interactions.

2.5. Testing method to determine sensitivity and specificity of AFB1 test strips

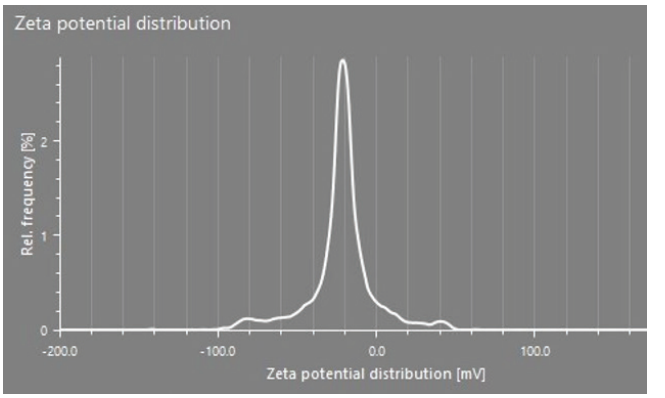
To assess the strip's diagnostic performance, 80 rice samples were tested: 40 AFB1-spiked positive samples (5 ng/ml) and 40 blank samples. Samples were ground, extracted with 70% methanol, evaporated, and reconstituted in borate buffer. Test strips were dipped into the prepared samples, and results were read visually after 5–10 minutes.

Sensitivity = (True Positives / Total Positive Samples) × 100%

Specificity = (True Negatives / Total Negative Samples) × 100%

3. RESULTS AND DISCUSSION

3.1. Antibody–Gold Nanoparticle Conjugation Efficiency



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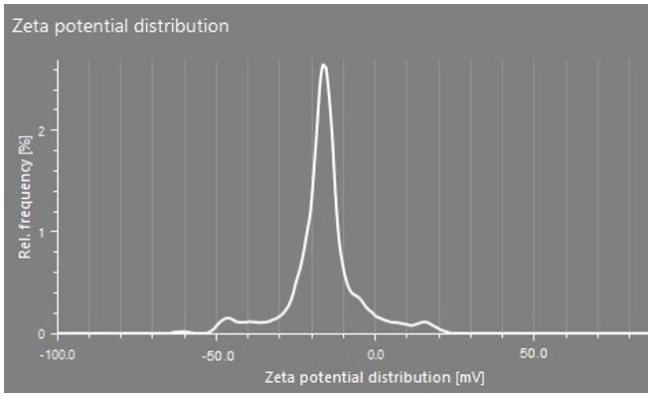

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Figure 2. Zeta potential measurements of gold nanoparticles (A) before and (B) after antibody conjugation.

Observation: The surface charge shifted from -21.8 mV to -15.4 mV after conjugation, confirming successful antibody adsorption onto the nanoparticle surface.

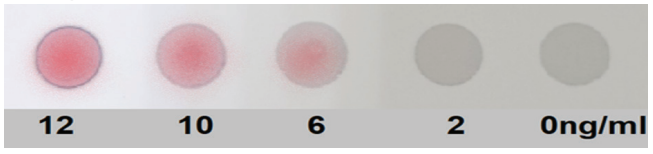

Figure 3. Dot blot assay for antibody activity after conjugation

Observation: Antibody retained reactivity toward AFB1, with the strongest signal at 12 ng/ml and a weak signal at 6 ng/ml, confirming preserved antigen-binding ability.

3.2. Detection Limit of the Test Strip

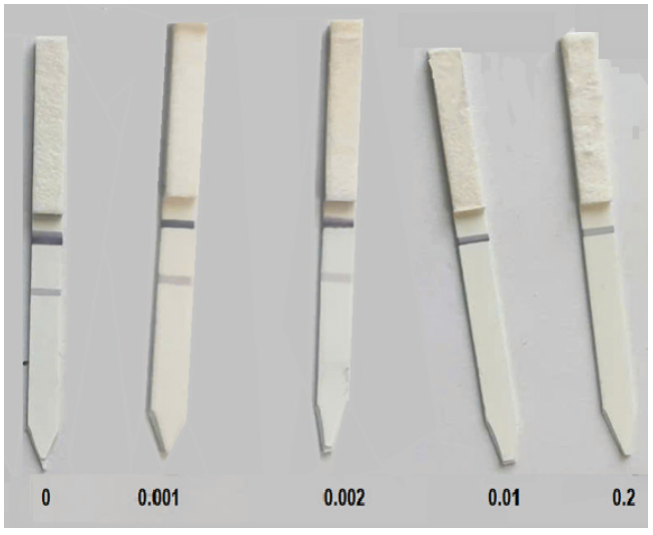

Figure 4. Detection limit evaluation.

Observation: The visual detection limit was 0.01 µg/ml. At 0.002 µg/ml, faint signals were observed. The effective detection range was 0.001–0.01 µg/ml.

Table 1. AFB1 Detection Reproducibility

AFB1 Concentration (ng/ml)	Trial 1	Trial 2	Trial 3	Trial 4	Trial n 5
0	-	-	-	-	-
1	-	-	-	-	-
2	+	+	+	+	+
10	+	+	+	+	+
200	+	+	+	+	+

Observation: All five replicates yielded positive results at 2 ng/ml, establishing this as the test strip's reliable detection threshold.

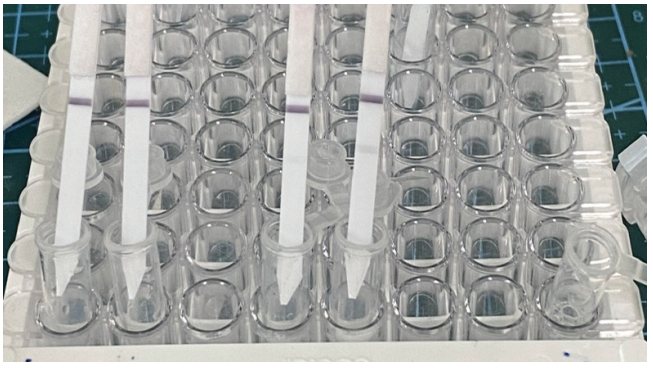

Figure 5. Dipstick test with replicates

3.3. Cross-Reactivity with Other Mycotoxins

Table 2. Cross-Reactivity Testing

Toxin	Testing (ng/ml)			
	1	5	10	50
Aflatoxin M1	-	-	-	-
Ochratoxin A	-	-	-	-
Patulin	-	-	-	-

Observation: No cross-reactivity was observed with the tested mycotoxins, confirming high specificity.

3.4. Sensitivity and Specificity Assessment

Table 3. Sensitivity and Specificity Assessment

Sample	Number	Result	
		Positive	Negative
AFB1-spiked	40	35	5
Blank	40	2	38
Total	80	37	3

Observation: The test strip demonstrated 87.5% sensitivity and 95% specificity under controlled lab conditions.

4. DISCUSSION

4.1. Antibody–Nanoparticle Conjugation

In this study, the conjugation of anti-AFB1 monoclonal antibodies to gold nanoparticles was achieved via passive adsorption, a commonly used approach in lateral flow assay development due to its simplicity, cost-effectiveness, and minimal reagent requirement. The physical adsorption mechanism relies on non-covalent interactions, including hydrophobic interactions, ionic bonds, and van der Waals forces. This method is, however, sensitive to physicochemical conditions such as pH, temperature, ionic strength, and stirring rate. Therefore, meticulous optimization is required to ensure stable conjugation and preservation of antibody bioactivity.

Our Zeta potential measurements confirmed successful conjugation, as the surface charge of gold nanoparticles changed significantly from -21.8 mV to -15.4 mV after antibody immobilization, indicating electrostatic surface modification attributable to protein adsorption. This shift is a reliable indicator of successful antibody binding to the nanoparticle surface.

Dot blot analysis further demonstrated that the antibodies retained their antigen-binding activity post-conjugation. The signal remained strong at 12 ng/ml of AFB1 and was still detectable at 6 ng/ml, suggesting that the biological functionality of the antibody was preserved. This is a critical factor in assay performance because any loss of antibody activity during the conjugation process could result in reduced sensitivity or false-negative outcomes. The use of borate buffer at pH 7.2 provided a near-physiological environment that supported protein stability and maintained the correct folding of the antibody during the conjugation procedure.

Thus, the physical adsorption strategy employed here, while inherently less stable than covalent binding, was effective in producing a functional conjugate for use in competitive lateral flow immunoassays.

4.2. Limit of Detection of the Dipstick Assay

The limit of detection (LOD) is a vital analytical parameter that determines the minimum concentration of a target analyte that can be reliably distinguished from background noise. In this study, the LOD of the AFB1 test strip was established at 2 ng/ml (0.002 µg/ml) based on consistent positive signals observed across five replicate tests. At lower concentrations (e.g., 1 ng/ml), the test line produced no visible signal, while 2 ng/ml yielded strong, reproducible colorimetric responses.

This detection limit is within the acceptable range for international food safety standards, including those set by the European Union (2 µg/kg for AFB1 in foodstuffs), indicating that the developed dipstick is appropriate for preliminary screening in practical food safety applications. The narrow detection range of 0.001–0.01 µg/ml further highlights the strip's ability to discriminate low levels of contamination, which is essential for early detection and risk prevention.

The observed inverse relationship between AFB1 concentration and test line intensity conforms to the principles of competitive immunoassays, wherein increased analyte levels result in decreased color development at the test line. These results confirm the analytical validity of the assay's design.

4.3. Cross-Reactivity Analysis

High assay specificity is crucial for accurate immunodiagnostics, particularly in complex food matrices that may contain structurally similar compounds. Cross-reactivity can lead to false-positive results and diminish the reliability of the test. In this study, we assessed potential cross-reactivity with other common mycotoxins-Aflatoxin M1 (AFM1), Ochratoxin A (OTA), and Patulin-across a concentration range of 1–50 ng/ml.

The results demonstrated no observable cross-reaction with any of the tested compounds, confirming that the anti-AFB1 monoclonal antibody used in this assay exhibits excellent specificity. This selective recognition of AFB1, without interference from other fungal toxins, reflects the robustness of the immunochemical design and ensures that the presence of structurally related mycotoxins does not compromise test results.

Such high specificity is critical for on-site screening tools, as it ensures accurate assessment even in complex or processed food matrices that may harbor multiple contaminants or matrix interferences. The ability of the assay to maintain high specificity in the presence of other mycotoxins enhances its value as a reliable tool for food safety screening in resource-limited or field conditions[10].

4.4. Sensitivity and Specificity of the AFB1 Test Strip

Sensitivity and specificity are key performance metrics for any diagnostic assay. Sensitivity refers to the ability of the test to correctly identify true positive samples, while specificity measures its ability to classify true negatives correctly. In our evaluation involving 80 rice samples, the test strip demonstrated a sensitivity of 87.5%, with 35 out of 40 AFB1-positive samples correctly identified. Specificity was 95%, with 38 out of 40 negative

samples producing accurate results.

These results indicate that the test strip offers high diagnostic accuracy, with minimal rates of false positives (5%) and false negatives (12.5%). The observed discrepancies may be attributed to several factors, such as inconsistent sample application, uneven antibody distribution on the membrane, or environmental influences (e.g., humidity, temperature) affecting assay performance. While these limitations suggest opportunities for process refinement, they do not significantly detract from the test's utility as a rapid screening tool.

It is worth noting that although the sensitivity of the LFIA dipstick is somewhat lower than that of gold-standard methods like ELISA or HPLC, its operational simplicity and rapid turnaround (5–10 minutes) make it a highly attractive option for field applications. The dipstick can serve as a first-line screening device to identify samples requiring confirmatory testing via more sophisticated analytical techniques.

Moreover, in high-AFB1 contamination scenarios, such as those encountered in post-harvest storage or transport, the dipstick provides a fast and actionable diagnostic signal that can guide immediate risk mitigation decisions. Its portability and user-friendliness also make it suitable for decentralized monitoring programs in remote or under-resourced areas.

5. CONCLUSION

This study successfully developed a competitive LFIA dipstick for rapid AFB1 detection. The assay allows visual interpretation within 5–10 minutes, with straightforward operation and good storage stability. Its practicality and effectiveness suggest strong applicability for on-site food safety surveillance, especially in remote or field-based scenarios.

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