Original Research

INITIAL STEPS OF RESEARCH INDEVELOPING A LATERAL FLOW IMMUNOASSAY KIT FOR RAPID DETECTION OF OCHRATOXIN A IN FOOD

Tong Duc Minh[1,✉], Nguyen Van Chuyen[1], Nguyen Van Ba[1], Nguyen Thi Thu Trang[1], Le Tuan Anh[1], Hoang Thi Truong[1], Vu Thi Hoa[1], Nguyen Trong Dat[2], Bui Khanh Toan[3]

[1] *Vietnam Military Medical University*
[2] *Military Insitute of Preventive Medicine, Vietnam*
[3] *Tay Nguyen Institute of Hygiene and Epidemiology, Vietnam*

ABSTRACT

Aims: To determine some technical parameters of rapid test strips to detect ochratoxin A (OTA).

Methods: The study was conducted at the Department of Military Hygiene, Military Medical Academy. We had optimized a number of test parameters including: pre-treatment of conjugate pads, antibody concentration covering the gold nanoparticles, capture antibody concentration, and running buffer. The technical parameters of test strips manufactured were determined to detect OTA, including cross-reactivity, stability, detection rates of positive and negative samples.

Results: Antibody concentration of 20 µg/ml covering the gold particle reached saturation state and the limit of determination was 1 µg/ml. The capture antibody concentration was 0.75 mg/ml; Lactose 10% and sucrose 10% enhanced the release of gold particle-antibody complexes through the conjugate membrane. The test strip did not cross-react with standard samples of aflatoxin B1, aflatoxin M1, and patulin at the concentrations tested. The detection rates of positive and negative samples were 80% and 92%, respectively.

Conclusion: The results showed that optimizing the parameters are important. Evaluation of the technical parameters of the rapid test strip for OTA detection showed that the test strip had high detection rates of positive and negative samples.

Keywords: *Immunochromatographic test strips, quick detection, ochratoxin A.*

I. INTRODUCTION

Ochratoxin A is a mycotoxin, primarily produced by species of the fungi Aspergillus and Penicillium [1]. The International Agency for Research on Cancer has classified ochratoxin A as a Group 2B carcinogen [2]. Ochratoxin A (OTA) is a contaminant found in various food products, such as cereals, coffee, dried fruits, and spices. If humans consume food contaminated with OTA, it can pose a serious health risk [3]. To protect human health, it is essential to detect food contaminated with mycotoxins.

Various analytical methods, such as high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS/MS), gas chromatography-mass spectrometry (GC-MS), and enzyme-linked immunosorbent assay (ELISA), have been utilized for the analysis of Ochratoxin A [4].

✉ Corresponding author: Tong Duc Minh
Email: minhhoa142@gmail.com
Doi: 10.56283/1859-0381/651
Submitted: 18/10/2023 Revised: 11/11/2023
Accepted: 25/11/2023
Published online: 28/11/2023

The methods mentioned above are time-consuming, require expensive equipment, and demand experienced technicians for operation. These techniques are not suitable for on-site diagnosis. Lateral flow immunoassay (LFIA) are gaining increasing attention for overcoming these issues. LFIA has the ability to detect mycotoxins with low cost, quick results, and user-friendliness. In particular, LFIA has been used for quantitative analysis by utilizing magnetic nanoparticles and a reading device.

Although LFIA has been widely applied, its sensitivity is limited. The objective of this study is to develop a high-sensitivity LFIA for detecting the mycotoxin Ochratoxin A by optimizing key parameters, including pre-treatment of conjugate pads to enhance the efficiency of releasing gold nanoparticles-antibodies, the antibody coverage concentration on gold nanoparticles, the antibody capture concentration, and the sample diluent. Through this optimization, we aim to lower the detection limit of the kit and achieve high sensitivity, specificity, and stability of the test strip.

II. METHODS

2.1. Chemicals

Two anti-Ochratoxin A antibodies (monoclonal antibody, mab0029-P from Cova lab and BP666 from Biotrend) were used to target the ochratoxin A antigen (01877 from Sigma). The aflatoxin B1, aflatoxin M1, and patulin antigens were purchased from Biotrend. IgG antibodies against mouse (M5899), bovine serum albumin (BSA), borate buffer, Tris-buffered saline (TBS), phosphate-buffered saline (PBS), Tween 20, sucrose, and lactose were obtained from Sigma, Germany. The gold nanoparticles 20nm (ab269935) were purchased from Abcam. All types of membranes were acquired from Whatman, including nitrocellulose membranes (FF120HP from Whatman, USA), composite membranes (Standard 17 from Whatman, USA), sample pad membranes, and absorbent pad membranes (CF4 from Whatman, USA).

2.2. Principles of the method

The immunochromatographic test strip, as shown in Figure 1, consists of four components: the sample pad, the gold-antibody conjugate, the nitrocellulose membrane, and the absorbent pad.

Sandwich mechanism: Single antibodies are used to bind to the gold nanoparticles conjugate. If the sample contains the antigen to be detected, the antibody will bind to the antigen, forming a complex of gold nanoparticle-antigen-antibody. This complex migrates to the nitrocellulose membrane and is then captured by another specific antibody that has been coated on the detection line. The presence of the detection line indicates a positive reaction. If there is no antigen in the sample, the test strip will only show a control line, indicating a negative sample.

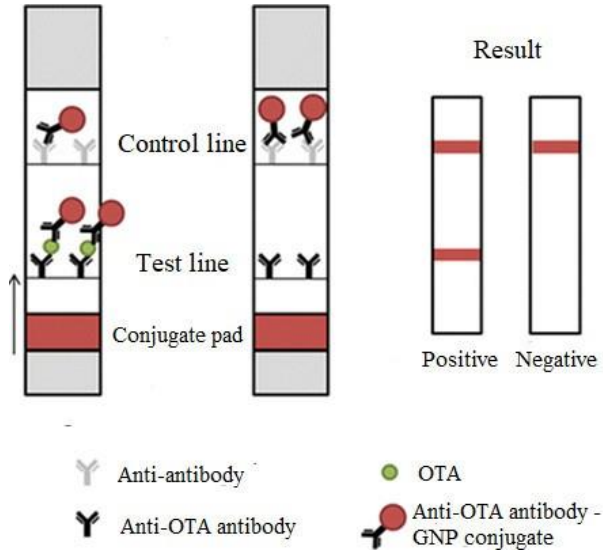

Figure 1. *Illustration of the immunochromatographic lateral flow strip test.*

2.3. Optimization of test tarameters

2.3.1. Optimization of antibody adsorption concentration on gold nanoparticles

The attachment of OTA antibodies to gold nanoparticles plays a crucial role in enhancing the sensitivity of the test strip. Gold nanoparticles that are bound with the antibodies should have high affinity for the analyte and low nonspecific binding to the nitrocellulose membrane. To achieve this goal, it's necessary to investigate the concentration of the recognition antibodies on the gold nanoparticles.

The intensity of the color signal on the test line of the test strip is directly proportional to the concentration of antibodies coated on the surface of the gold nanoparticles. Therefore, the amount of antibodies bound to the gold nanoparticles needs to be optimized. When the quantity of antibodies added to the gold nanoparticle solution increases, the number of antibody molecules covering the gold nanoparticles also increases. However, once the antibodies are saturated on the gold nanoparticles' surface, no further binding can occur. Therefore, different amounts of single OTA antibodies were added at concentrations of 5 µg/ml, 10 µg/ml, 15 µg/ml, 20 µg/ml, 25 µg/ml, and 30 µg/ml in a 50 µl gold nanoparticle solution. The assessment criteria were based on observing which sample achieved the best results, which was the sample with the darkest color signal on the test line when tested with a positive sample.

2.3.2. Optimization of the antibody on the test line concentration

Optimizing the concentration of the detection antibodies on the test line is necessary because a low concentration of capture antibodies results in fewer gold nanoparticle-antibody-antigen complexes being retained on the detection line. This leads to a weaker color signal on the test line. Conversely, a high concentration of capture antibodies results in more complexes being retained on the test line, leading to a stronger color signal. To optimize the concentration of single OTA antibodies on the test line, we diluted the antibodies as follows: 1.0 mg/ml, 0.75

mg/ml, 0.50 mg/ml, and 0.25 mg/ml. The evaluation was conducted by observing the results after 10 minutes with the naked eye. The clearest color signal on the detection line was observed when testing with positive samples.

2.3.3. Optimization of the release of gold nanoparticle-antibody complexes on the composite membrane

The treatment of the composite membrane is crucial to ensure the consistent and uniform release of gold nanoparticle-antibody complexes. Previous studies have demonstrated that the addition of sugar to the buffer solution creates favorable conditions for releasing gold nanoparticle-antibody complexes from the composite membrane. Therefore, the chosen sugar concentrations for testing were lactose 20%, sucrose 20%, lactose 10%, and sucrose 10%. The evaluation was based on the efficiency of releasing the gold nanoparticle-antibody-antigen complexes. The highest efficiency of complex release led to the retention of more complexes on the test line and the strongest color intensity.

2.3.4. Optimization of sample pad buffer solution

The dilution buffer for the antigen is an important component for reducing nonspecific binding. A solution to minimize nonspecific binding is to find a dilution buffer with a pH that matches the protein's isoelectric point. BSA is used as a blocking agent to reduce nonspecific binding, while Tween 20 acts as a surfactant to eliminate nonspecific binding due to hydrophobic interactions.

The antigen dilution buffer consists of 0.5% (v/v) Tween 20 and 0.5% (w/v) BSA, prepared with various buffer solutions to select the optimal buffer. The tested buffer solutions include: PBS (10 mM, pH 7.4); Borate (10 mM, pH 7.2); Borate (50 mM, pH 9.0); Tris (50 mM, pH 8.0); MES (50 mM, pH 6.5); Tris buffer (70 mM, pH 7.0). The evaluation criteria were based on the intensity of the color signal on the test line, with the densest color observed in the positive sample and the weakest signal in the negative test, when the buffer was introduced into the test strip.

2.4. Cross-reactivity

The test strip was tested with antigen samples from different mycotoxins such as aflatoxin B1, aflatoxin M1, and patulin at concentrations ranging from 2 to 100 µg/ml. Cross-reactivity occurred when the test results were positive for these mycotoxins, and no cross-reactivity was observed when the results were negative.

2.5. Evaluation of the detection rate with samples containing OTA at a standard concentration of 5 µg/ml and samples without OTA

The test was conducted on 25 samples containing OTA at a standard concentration of 5 µg/ml and 25 samples without OTA. The detection rates of positive samples and negative samples were calculated using the following formulas:

$$+ \text{Detection rate of positive samples} = \frac{\text{number of test strips with positive results}}{\text{number of samples with 5 µg/ml OTA}}$$

$$+ \text{Detection rate of negative samples} = \frac{\text{number of test strips with negative results}}{\text{number of samples without OTA}}$$

2.6. Determining the stability of the test strip

The stability of the competitive immunochromatographic test strip: storing the test strips under different temperature conditions (40 °C, 25 °C, and 37 °C) while sealed in airtight bags with silica gel desiccants to prevent moisture exposure.

The expiration date of the results was determined by storing the test strips for various periods. Subsequently, the color changes of the test lines were observed when testing positive samples on days 30, 60, and 90. The reliability of the testing was established by comparing the test strip results with both negative and positive samples. The evaluation of the corresponding results was based on the color intensity on the test line when testing positive samples. Each parameter was repeated five times to assess repeatability.

III. RESULTS

3.1. Optimization of antibody adsorption concentration on gold nanoparticles

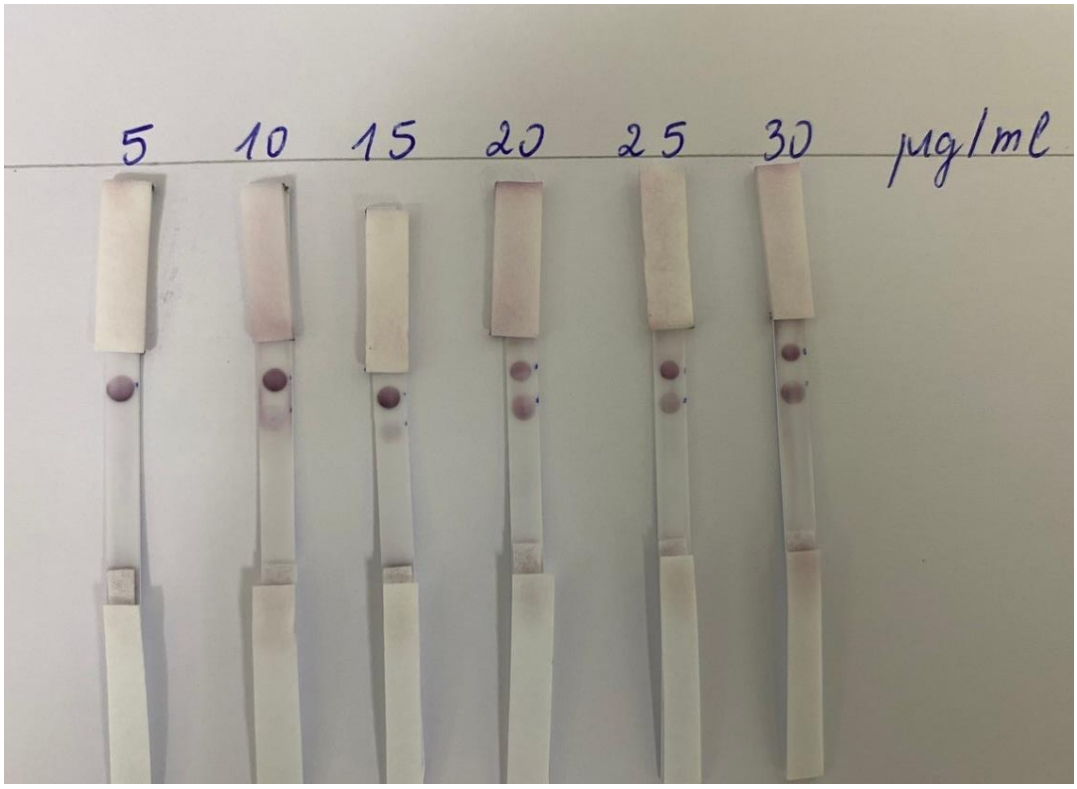

Figure 2. *Construction of the sandwich immunoassay test strip with different concentrations of ochratoxin a antibodies attached to gold nanoparticles at an antigen concentration of 1 µg/ml.*

We conducted antigen testing for OTA at concentrations of 0 µg/ml, 0.5 µg/ml, 1 µg/ml, 2 µg/ml, and 4 µg/ml. The color signal intensity on the test line was directly proportional to the antibody concentration. The research results showed that the color signal intensity was the highest when using an antibody

concentration of 20 µg/ml with an additional antigen concentration of 1 µg/ml. However, at an antigen concentration of 0.5 µg/ml, none of the five test strips displayed a detection line. Lower antibody recognition concentrations resulted in weaker color signals on the test line. The findings indicated that when using antibody concentrations of 20 µg/ml, 25 µg/ml, and 30 µg/ml, the color signals on the test line were equivalent. Therefore, an antibody concentration of 20 µg/ml was chosen for further testing. The detection limit of the test strip was 1 µg/ml.

3.2. Optimization of the antibody on the test line concentration

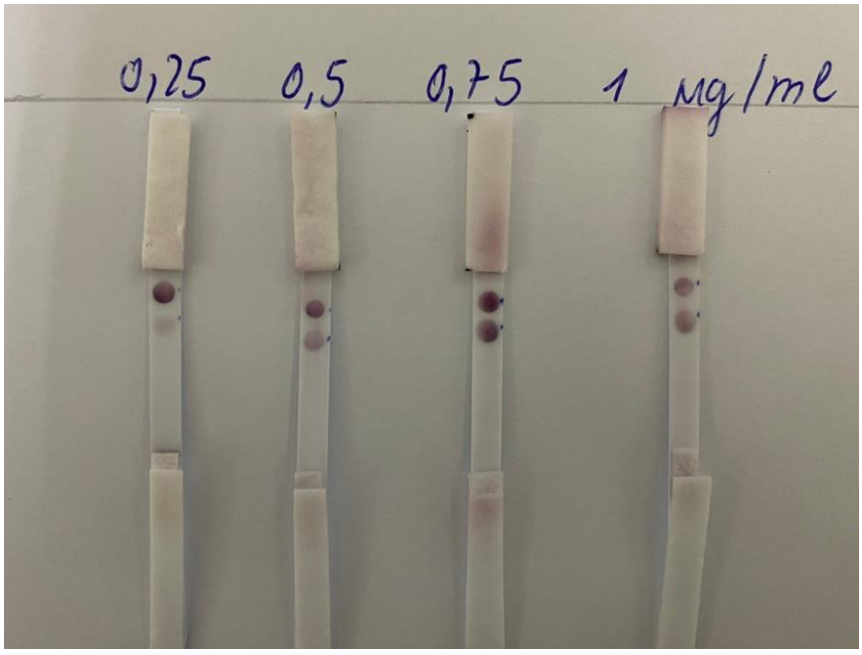

Figure 3. *Selection of the antibody concentration on the detection line.*

The results of the continuous dilution of the capture antibody concentration are presented in Figure 3. A concentration of 0.75 mg/ml was considered the optimal concentration for the assay. The signal intensity observed at this concentration was stronger compared to samples with concentrations of 1 mg/ml, 0.5 mg/ml, and 0.25 mg/ml.

3.3. Optimization of the release of gold nanoparticle-antibody complexes on the composite membrane

When dripping the buffer solution onto the conjugate membranes (1), (2) and (3) (Figure 4), most of the gold-particle-antibody complexes in sample 3 were completely released from the conjugate membrane. Therefore, the remaining color on the conjugate membrane wasthe faintest. Additionally, the results on the test strip indicated that the color intensity in sample 3 was the strongest. In contrast, (1) had a lower efficiency in releasing gold-particle-antibody complexes compared to (2), hence the color signal on the test line of (1) was lighter than (2).

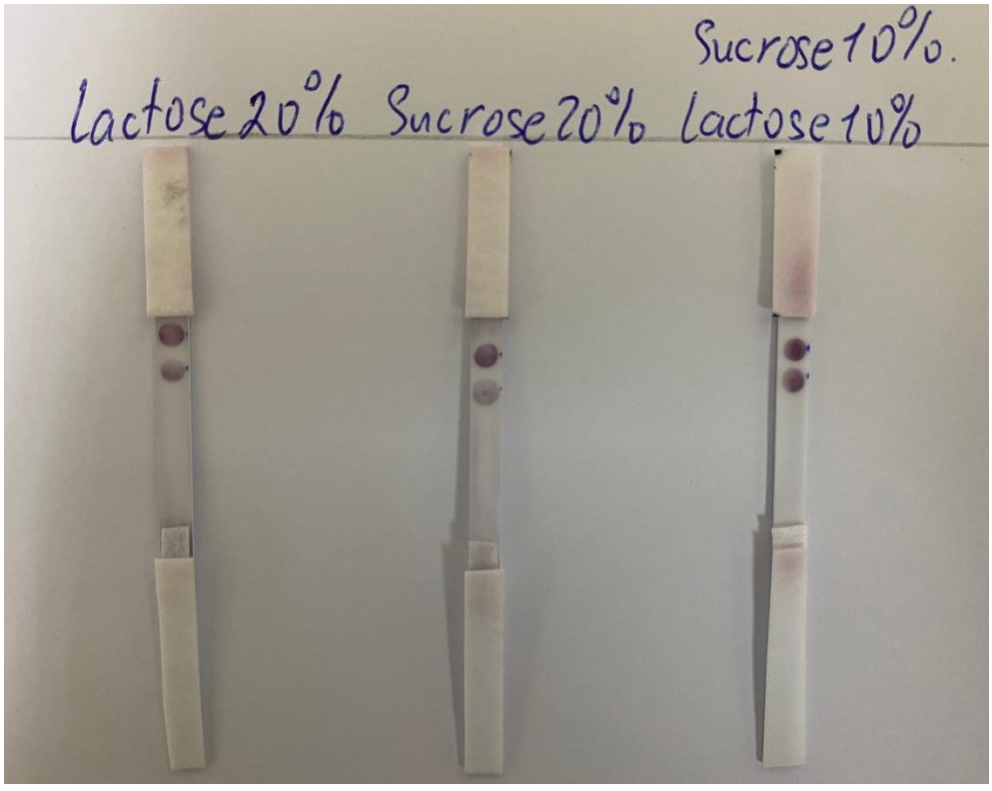

Figure 4. *Observation of color intensity on the test line when the conjugate membrane is treated with lactose 20% (1), sucrose 20% (2), lactose 10% and sucrose 10% (3).*

3.4. Optimization of sample pad buffer solution

The positive samples were diluted with 6 different buffer solutions. Figure 5 (a) shows that when using MES buffer (50 mM, pH 6.5), the color signal on the test line was the strongest. Tris buffer (70 mM, pH 7.0) had the second-strongest color signal, followed by Tris buffer (50 mM, pH 8.0).

The negative samples tested in Figure 5 (b) showes that when using MES buffer (50 mM, pH 6.5), Tris buffer (70 mM, pH 7.0), and Tris buffer (50 mM, pH 8.0), no false positive signals were observed. In contrast, when using PBS buffer (10 mM, pH 7.4) and borate buffer (10 mM, pH 7.2), there was a false positive signal in one negative sample.

The research results indicate that MES buffer (50 mM, pH 6.5) enhances the sensitivity of the test strip because it has the strongest color signal and no false positive signals.

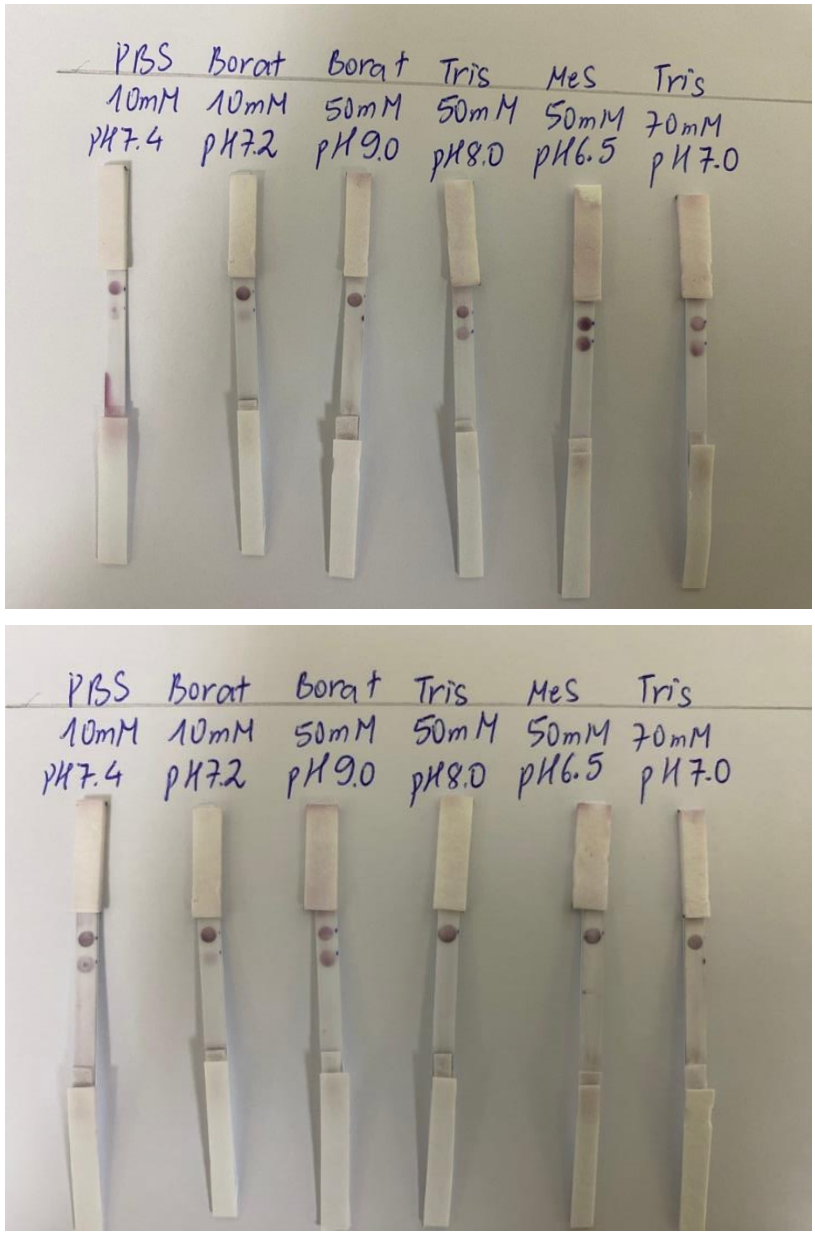

Figure 5. *(a) Test results on positive samples when using 6 types of antigen dilution buffer. (b) Results when testing negative samples.*

3.5.Cross-Reactivity

Table 1 shows that the test strip did not exhibit cross-reactivity with standard samples of aflatoxin B1, aflatoxin M1, and patulin at the tested concentrations.

Table 1. *Cross-reactivity testing with standard samples of aflatoxin B1, aflatoxin M1, and patulin.*

Toxin	Concentration (µg/ml)			
	2	10	50	100
Aflatoxin B1	-	-	-	-
Aflatoxin M1	-	-	-	-
Patulin	-	-	-	-

3.6. Sensitivity and specificity

Table 2. *Testing the ability to detect OTA toxin in samples at various concentrations.*

Sample	*n*	Positive	Negative
Sample containing OTA toxin (5 µg/ml)	25	20	5
Sample without OTA toxin	25	2	23
Total	50	22	28

The test results for the OTA-positive samples used for testing in Table 2 are as follows: OTA was detected positively in 20 out of 25 OTA-positive samples using the LFIA test, with a positive value of ≥ 5 µg/ml - equivalent to the positive value of the milk sample detected by HPLC. Among the 25 OTA-negative samples analyzed, 2 samples were false positives, and 23 samples were accurately detected as negative.

The rapid test's sensitivity was 80% (20/25 samples), while its specificity was 92% (23/25 samples).

3.7. The stability of the test strip

Table 3. *Results of the test strip's stability when stored at different temperatures.*

Duration (day)	False negative (%)	False positive (%)	Qualified samples	Color intensity on the test line
4 °C				
30 d	0	0	5/5	++
60 d	0	0	5/5	++
90 d	0	0	5/5	++
25°C				
30 d	0	0	5/5	++
60 d	0	0	5/5	++
90 d	0	0	5/5	++

Duration (day)	False negative (%)	False positive (%)	Qualified samples	Color intensity on the test line
37 ^{o}C				
30 d	0	0	5/5	++
60 d	0	0	5/5	+
90 d	0	0	5/5	+

Note: "++" indicates strong red color intensity. "+" indicates light color intensity. "-" indicates no color.

The results of the test strip's stability are shown in Table 3. The sensitivity of the test strip remains unchanged after 90 days at both 40°C and 25°C. The color signal on the test line is strong when testing positive samples, and there are no false positive or false negative results. When stored at 37°C for 60 days, the color signal becomes faint when testing positive samples. Similarly, after 90 days of storage, the color signal on the test line continues to weaken in positive sample testing. However, no false negative or false positive results were observed during the storage period.

IV. DISCUSSION

4.1. Optimization of antibody adsorption concentration on gold nanoparticles

The combination of antibodies and gold nanoparticles is the result of electrostatic attraction. Under certain conditions, gold nanoparticles have a negative surface charge, while antibodies have a positive surface charge [5]. Gold nanoparticles with a negative charge combine with antibodies carrying a positive charge, resulting in a clear color signal intensity on the test line (Figure 2). This confirms that the antibody molecules have successfully been conjugated with the gold nanoparticles.

Monoclonal antibodies attached to gold nanoparticles play a crucial role in capturing OTA antigens in the sample. The antibody concentration is closely related to the color signal on the test strip. The concentration of monoclonal OTA antibodies conjugated with gold nanoparticles should be at its highest to enhance the efficiency of antigen binding, thus increasing the sensitivity of the test strip. To determine the appropriate amount of antibody immobilized on the gold particles, we investigated six concentrations: 5 µg/ml, 10 µg/ml, 15 µg/ml, 20 µg/ml, 25 µg/ml, and 30 µg/ml. The results shows that when the antibody concentration was 20 µg/ml, the color signal on the test line was the strongest. As the antibody coverage on the gold particles decreased, the color signal decreased. At antibody concentrations of 25 µg/ml and 30 µg/ml, the color signal was equivalent to that at 20 µg/ml. From this, we can conclude that at a concentration of 20 µg/ml, the antibody coverage on the gold nanoparticles reaches saturation. In the same experiment, at an antibody concentration of 20 µg/ml, the detection limit was 1 µg/ml.

4.2. Optimization of the antibody on the test line concentration

There is a correlation between the color signal on the test line and the amount of antibody immobilized on the detection line. The color signal increases as the antibody concentration at the detection line increases. This is due to the enhanced interaction between the gold nanoparticle-antibody-antigen complex and the antibody immobilized at the test line. Because a higher amount of immobilized antibody captures more antigens, it increases the recognition of the antigen, thereby increasing the number of antibody-antigen-antibody-gold nanoparticle complexes formed on the test line.

The LFIA test strips with immobilized capture antibodies on the test line are used to evaluate the color intensity at the test line. The highest color signal intensity is observed at an antibody concentration of 0.75 mg/ml. The color intensity observed is the same at antibody concentrations of 1 mg/ml and 0.5 mg/ml. Therefore, the concentration of 0.75 mg/ml is chosen on the conjugate membrane because it is cost-effective and ensures the detection of mycotoxins.

4.3.Optimization of the release of gold nanoparticle-antibody complexes on the composite membrane

Consistent and uniform release of the gold nanoparticle-antibody-antigen complexes is essential to ensure the smooth flow of the sample towards the nitrocellulose membrane, avoiding clogging and enhancing sensitivity. Our research results show that a 10% lactose and 10% sucrose buffer enhances the release of the gold nanoparticle-antibody complexes through the conjugate membrane compared to using a lower sugar concentration. This result aligns with previous studies that found optimal complex release at a sugar concentration of 20% with equal ratios of lactose and sucrose [6]. Furthermore, when samples were processed with a higher sugar concentration, we observed a stronger color signal on the test line compared to the lower sugar concentration. This might be due to the higher sugar concentration increasing the viscosity of the flow, resulting in reduced flow velocity and, in turn, extending the interaction time between antibodies and antigens. This makes the immune reaction on the test line easier, leading to a more intense color signal.

4.4. Optimization of sample pad buffer solution

During our experiments with the test strips, we observed that a small amount of gold nanoparticle-antibody-antigen complexes could not be released from the conjugate membrane or became trapped at the initial position of the membrane. As a result, this could decrease the signal on the test line because the complexes cannot undergo immune reactions at the test line. Furthermore, if the dilution buffer for the antigen is not appropriate, it could alter the total surface charge of the gold nanoparticles, leading to false positive results in negative samples. To address this issue, the dilution buffer for the antigen has been optimized.

BSA is added to the dilution buffer to reduce non-specific binding of antibodies

to the membrane. Tween 20 makes the gold nanoparticle-antibody complex hydrophilic, prevents agglomeration, and simultaneously enhances the efficiency of releasing the gold nanoparticle-antibody complex into the flow. The optimal dilution buffer in our study is MES 50 mM, pH 6.5; BSA 0.5%; Tween 20 0.5%. The current research has identified an appropriate sample dilution buffer that enhances the antigen-antibody reaction, thereby increasing the sensitivity of the test strip.

4.5. Cross-reactivity testing with standard samples of aflatoxin B1, aflatoxin M1, and patulin

We examined the cross-reactivity of the rapid test strip, specifically testing for mycotoxins other than OTA, which are also produced by the *Aspergillus* fungus. Aflatoxin B1 is a mycotoxin produced by *Aspergillus* and is commonly found in cereal products. Aflatoxin M1 is a metabolite of aflatoxin B1 and is often present in various dairy products. In our study, we determined the cross-reactivity of the test strip with aflatoxin B1, aflatoxin M1, and patulin in concentration ranges from 2 to 100 µg/ml. The results showed no cross-reactivity with standard samples of AFB1, AFM1, and patulin at the tested concentration ranges.

4.6.Determine the sensitivity and specificity of the OTA test kit

Although the test strip's sensitivity may be lower than that of ELISA or HPLC, the kit offers a short processing time. Preliminary assessments can be made within 5 minutes, and results can be read within a 10 to 15-minute time frame. With this timeframe, the test strip can be used for screening the contamination levels, allowing testers to make initial evaluations, especially in cases of high OTA contamination in food.

4.7. The stability of the test strip

The stability of the test strip was evaluated by testing positive samples on days 30, 60, and 90 from the day of gold nanoparticle-antibody spraying on the conjugate membrane. When testing positive samples, Table 1 shows that the color signal is strong when the test strip is stored at temperatures of 40°C and 25°C for 3 months. When stored at 37°C, the color signal performs well and remains stable for 1 month, with a slight reduction in color signal intensity after 2 to 3 months.

Based on the research results, we conclude that the test strip can be stored at 40°C and 25°C for up to 3 months and can further extend the storage time to evaluate stability. At a storage temperature of 37°C, after 2 months of storage, there is a slight reduction in the color signal on the test line.

V. CONCLUSION

In this study, we have developed a lateral flow immunoassay (LFIA) test strip for the rapid detection of OTA. The results show that it is crucial to optimize parameters, such as the antibody concentration absorbed on gold nanoparticles, antibody concentration sprayed on the test line, releasing the gold nanoparticle-antibody complex on the conjugate membrane, and using the appropriate dilution buffer on the test strip. The limit of detection of OTA was 1 µg/ml. The test strip shows no cross-reactivity with standard samples of aflatoxin B1, aflatoxin M1, and patulin at the tested concentrations. The rapid test strip exhibits a sensitivity of 80% (20/25 samples) and a specificity of 92% (23/25 samples). We have demonstrated the stability of the test strip for 3 months when stored at 40°C and 25°C. Furthermore, we have enhanced the sensitivity of the LFIA test strip by optimizing the technical parameters.

References

1. Gao YN, Wu CQ, Wang JQ, Zheng N. Metabolomic Analysis Reveals the Mechanisms of Hepatotoxicity Induced by Aflatoxin M1 and Ochratoxin A. *Toxins* (Basel). 2022;14(2):141. doi:10.3390/toxins14020141.
2. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2002, Some traditional herbal medicines, some mycotoxins naphthalene and styrene. IARC Monogr. Eval. Carcinog. *Risks Hum*. 82:1–556
3. Stoev SD. Long term preliminary studies on toxic and carcinogenic effect of individual or simultaneous exposure to ochratoxin A and penicillic acid in mice. *Toxicon.* 2020;184:192-201.
4. Heussner AH, Bingle LE. Comparative Ochratoxin Toxicity: A Review of the Available Data. *Toxins* (Basel). 2015;7(10):4253-82. doi:10.3390/toxins7104253.
5. Kong D, Liu L, Song S, et al. A gold nanoparticle-based semi-quantitative and quantitative ultrasensitive paper sensor for the detection of twenty mycotoxins. *Nanoscale*. 2016;8:5245–5253.
6. Uri D. Paper-Based Lateral Flow Device for the Detection of I α IP via ELISA Open Access. Master's Thesis, University of Rhode Island, Kingston, RI, USA. 2014.