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Immunochromatographic Test Systems using Anti-Species Antibodies–Colloidal Gold Conjugate: Their Features and Benefits on the Example of Ochratoxin A Detection[1]

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Abstract—The traditional immunochromatographic assay using a conjugate of gold nanoparticles with specific ochratoxin A (OTA) antibodies and a new type of assay with indirect labeling using a combination of free antibodies and a conjugate of gold nanoparticles with anti-species antibodies were compared using the example of OTA detection. In the proposed assay, specific antibodies are included in the sample dilution buffer, which increases the duration of their interaction with the antigen, while a conjugate of anti-species antibodies with the marker is applied to the test strip. The assay was approbated for OTA detection in maize extracts. Transition to indirect labeling was shown to reduce the OTA detection limit by two orders of magnitude up to 0.12 ng/mL. The causes of this improvement are discussed. The high sensitivity of immunochromatography with indirect labeling makes it a promising approach for detection of various antigens with low molecular weight.

Keywords: immunoassay, test-strips, colloidal gold, antibody – nanoparticle complexes, mycotoxins

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Immunochromatographic assay (ICA) is widely used to screen samples for medical diagnostics and quality control of raw materials and food [1, 2]. The rapidity and ease of use of ICA are ensured by preliminary application of all assay reagents to the test strip membranes. Contact with the probe initiates all the necessary immunochemical reactions resulting in the staining of certain zones of the test strip, and in 10–15 min the assay results can be visualized or registered with an instrument. To detect compounds with low molecular weight, a competitive ICA format is used in which the antigen contained in the sample reacts with specific antibodies conjugated to the stained marker, most often with gold nanoparticles (GNPs), and prevents the marker from binding to the antigen immobilized in the assay zone of the test strip.

However, the development of highly sensitive systems according to this scheme is hampered by an internal contradiction: it is impossible to simultaneously ensure sufficient intensity of the assay signal (staining of the analytical zone) and high sensitivity of the assay since the signal is weakened at low concentrations of the detectable antigen. Thus, to improve the reliability of the assay, the concentration of conjugates of specific antibodies and GNPs should be as high as possible, and for highly sensitive detection, the concentration should be extremely low.

It should be noted that the conjugates used for ICA have tens to hundreds of antibody molecules per GNP. Therefore, when the antigen blocks only some of the antibodies on the surface of a nanoparticle, the conjugate retains its binding ability in the analytical zone. The intensity of staining decreases only when the antibodies are completely or almost completely blocked (i.e., at high antigen concentrations). In addition, a polyvalent conjugate is more likely to bind to polyvalent antigen sin the analytical zone than to free analytes in the sample, preventing effective competition.

To solve this problem, we proposed the use of specific antibodies and a marker as two independent reagents in the ICA [3]. ICA with indirect labeling uses free specific antibodies for competitive interaction and conjugates of anti-species antibodies and GNP to label the specific immune complexes that are formed.This replacement enables independent variation in the content of specific antibodies and the marker, providing optimal conditions for a highly sensitive and reliable assay. This approach allowed us to reduce the detection limit of aflatoxin B1 by 40 times

[1] The article was translated by the authors.

[3], that of zearalenone by 1500 times [4], and that of T-2 toxin by 6 times [5].

The present work implements a variant of ICA with indirect labeling characterized by the transfer of specific antibodies to the sample dilution buffer. Therefore, the interaction of antibodies and antigen occurs in a homogeneous solution (but not in the flow moving along the test strip) and reaches the equilibrium required for a highly sensitive assayin the minimum amount of time. The ICA was used to detect ochratoxin A (OTA). This mycotoxin (a metabolite of mold fungi such as Aspergillus and Penicillium) is a widespread toxic contaminant of agricultural crops (wheat, barley, rye, etc.) that negatively impacts human health [6–8]. The Commission Regulation (EC) no. 1881/2006, established on December 19, 2006, and the Technical Regulation of the Customs Union on the Safety of Grain (TR CU 015/2011) stipulated that a maximum of 5 ng/g of OTA could be present in grain. The importance of controlling OTA to ensure food safety necessitates rapid and highly sensitive methods for its detection.

EXPERIMENTAL PART

Materials and Reagents

In this work, hydrochloric acid, sodium citrate, bovine serum albumin (BSA), Triton X-100 and Tween-20 detergents, 3,3',5,5'-tetramethylbenzidine (TMB), and sodium azide were obtained from Sigma-Aldrich (USA). OTA was obtained from HromResurs (Russia). Monoclonal antibodies against OTA and OTA-BSA conjugate were obtained and characterized in previous studies by Kong et al. [9]. Goat antibodies against mouse immunoglobulins were obtained from Arista Biologicals (USA). Peroxidase-labeled goat antibodies against mouse immunoglobulins were provided by the Medgamal branch of the N.F. Gamaleya National Research Center for Epidemiology and Microbiology (Russia). Other reagents (solvents, buffer components, etc.) had a high purity grade.

The following buffers were used for the immunoassay:

PB—50 mM potassium phosphate buffer (PB) (pH 7.4) containing 100 mM sodium chloride;

PBTr—PB containing 0.05% Triton X-100;

PBTw—PB containing 0.05% Tween-20;

TBSA—10 mM Tris buffer (pH 8.5) containing 1% BSA, 1% sucrose, and 0.05% sodium azide; and

TTBSA—TBSA containing 0.05% Tween-20.

An enzyme-linked immunosorbent assay (ELISA) was performed in transparent 96-well polystyrene microplates with high sorption capacity (Corning, USA). The optical density of the products of the enzymatic reaction was measured using a Zenyth 3100 microplate reader (AnthosLabtec Instruments, Austria).

Hi-Flow Plus nitrocellulose membranes, absorbing membranes, and glass fiber membranes (Millipore, USA) were used to make the ICA systems. Immunoreagents were applied with an IsoFlow dispenser (Imagene Technology, USA). A Guillotine Cutter ZQ4200 (Shanghai Kinbio Tech, China) was used to cut the assembled multimembrane composites onto the test strips.

ELISA for OTA

The OTA-BSA conjugate was adsorbed in microplate wells at 4°C overnight at a concentration of 1 μg/mL from 100 μL of PB. After washing the wells four times with PBTr, 50 μL of OTA solution was diluted with PBTr to a concentration of 500–0.03 ng/mL. Then, 50 μL of the MAt solution in PBTr at a concentration of 300 ng/mL was added and the mixture was incubated for 1 hr at 37°C. The same washing procedure was repeated, and then 100 μL of peroxidase-labeled anti-species antibodies diluted with PBTr to 1 : 3000 were added to each well and incubated for 1 hr at 37°C. To determine the activity of peroxidase bound with the surface of the wells after washing with a solution of the substrate, 0.42 mM TMB and 1.8 mM H_2O_2 in 0.1 M sodium citrate buffer (pH 4.0, 100 μL per well) were added to the wells. After incubation for 15 min at room temperature, the reaction was stopped by addition of 50 μL of 1 M H_2SO_4. The optical density of the oxidation product of TMB was measured at 450 nm. The experiments were performed in triplicate.

The dependence of A_{450} on OTA concentration in the sample was approximated by the sigmoid 4-parameter function using Origin 7.5 software (OriginLab, USA). The detection limit, calculated as described in [10, 11], corresponded to a 10% decrease in A_{450}.

Synthesis of Conjugates of Antibodies with Gold Nanoparticles

GNPs with an average diameter of 30 nm were obtained by reducing chloroauric acid with sodium citrate using the Frens method [12] with some modifications [13].

A GNP solution with a concentration of 50 μg/mL (A_{520} = 1) was used for conjugation. The pH of the solution was adjusted to 8.9–9.0 by addition of 0.1 M sodium carbonate. Then, anti-species or OTA-specific antibodies were added to GNPs (10 μg per 1 mL). The resulting mixture was incubated for 45 min at room temperature. Then, a 10% aqueous BSA solution (V_{GNP} : V_{BSA} = 40 : 1) was added and the mixture was stirred vigorously for 15 min. The resulting conjugate was precipitated by centrifugation (15 min, 15000 *g*) at 4°C. The precipitate was re-dissolved in TBSA and the conjugate was stored at 4°C.

Production of Immunochromatographic Test Strips

A conjugate of GNPs with OTA-specific antibodies—A_{520} = 0.25 for the traditional assay scheme – or anti-species antibodies—A_{520} = 3 for the indirect labeling scheme—was applied to a glass fiber membrane. In both cases, the consumption of conjugate solution in TTBSA was 3.2 μL per 1 mm of membrane. A solution of OTA-BSA conjugate in PB at a concentration of 1 mg/mL was applied to the working nitrocellulose membrane. Its consumption was 0.1 μL per 1 mm of membrane. The working, glass fiber, and absorbing membranes were fixed on a plastic support and cut into strips that were 3.5 mm wide. The resulting test strips were stored at 20–22°C in a sealed foil-covered bag with silica gel.

Preparation and Characterization of Samples

The ground corn kernels were mixed with an extraction solution ($V_{methanol}$: V_{water} = 70 : 30) at a ratio of 1 : 5 and incubated with gentle stirring at room temperature for 30 min (a modified version of the process described in [14]). After 15 min of centrifugation at 15000 *g*, supernatants were collected and stored at 4°C.

Analysis of the resulting extracts was performed as described in [15] using a liquid chromatograph with a triple-quadrupole mass spectrometric detector (Agilent 6460, Agilent Technologies, USA) and demonstrated the absence of OTA. The OTA solutions were added to the extracts on the day of the immunoassay.

Conducting ICA (Traditional Scheme)

A sample (standard OTA solution or corn extract) and PBTw were mixed at a ratio of 1.0 : 2.5. A test strip taken out of the bag in a strictly vertical position was immersed 5–7 mm into the sample (100 μL) for 15 min at room temperature. Then, it was removed and placed on a horizontal surface. The experiments were performed in triplicate.

Conducting ICA (Indirect Labeling Scheme)

A sample (standard OTA solution or corn extract) and PBTw containing antibodies at a concentration of 125 ng/mL were mixed at a ratio of 1.0 : 2.5. A test strip was incubated with the sample as described above, after which it was removed and placed on a horizontal surface. The experiments were performed in triplicate.

Registration of ICA Results

Staining in the analytical zone was evaluated either visually or instrumentally by recording an image on a Lide 90 scanner (Canon) at a resolution of 600 dpi without automatic contrasting and color correction. The staining intensity was calculated using TotalLab v2.01 software (Nonlinear Dynamics, UK) in accordance with [16].

Approximation of the obtained concentration dependencies and calculation of the instrument detection limit for ICA were carried out in the same way as for ELISA (see above).

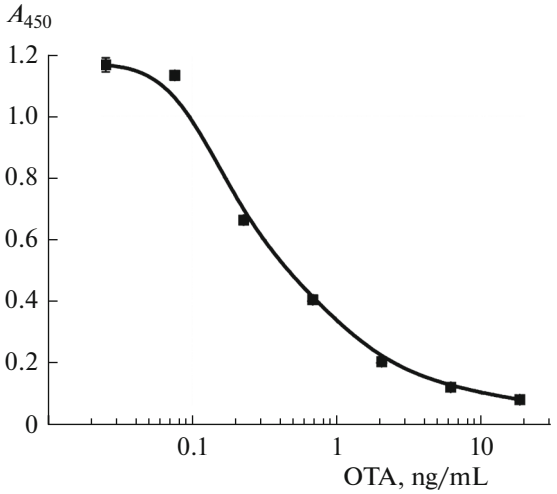

Fig. 1. The concentration dependence of OTA detection by ELISA.

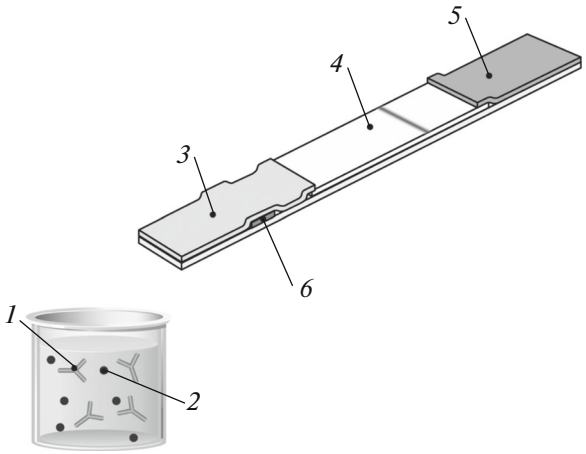

Fig. 2. The scheme of the developed analytical system, which consists of a well for sample dilution and a test strip. (*1*) specific antibodies against OTA; (*2*) OTA; (*3*) absorbing membrane for the sample; (*4*) working nitrocellulose membrane with applied OTA-BSA conjugate; (*5*) final absorbing membrane; (*6*) glass fiber membrane with applied anti-species antibodies–GNP conjugate.

RESULTS AND DISCUSSION

Characterization of Antibodies

Antibodies against OTA were preliminarily characterized by the ELISA method. The OTA detection limit was1 ng/mL (Fig. 1). The high affinity of these

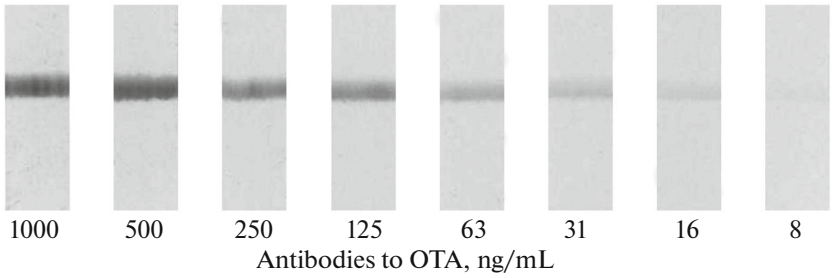

Fig. 3. Binding of the marker in the analytical zone of the test strip at different concentrations of antibodies against OTA.

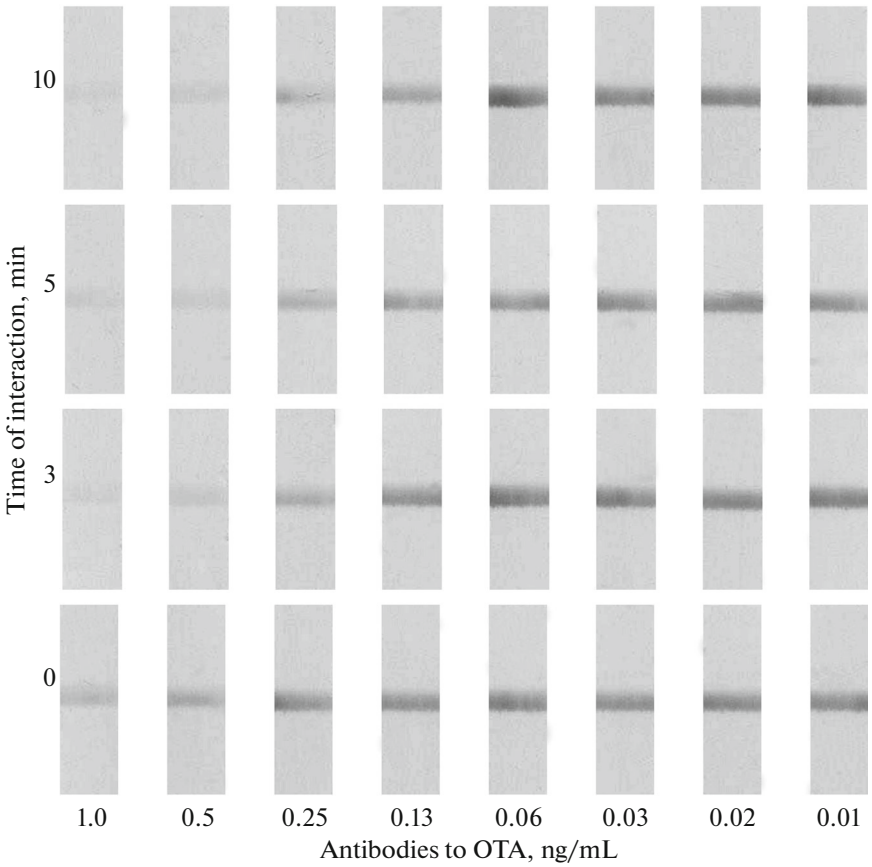

Fig. 4. The influence of different durations of interaction between the sample and specific antibodies on ICA results.

antibodies makes them suitable reagents for the ICA system that we are developing.

ICA Scheme with Indirect Labeling

For highly sensitive detection of OTA, antibodies were indirectly labeled as follows (Fig. 2). The OTA-BSA conjugate was applied and dried on a nitrocellulose membrane, whereas the conjugate of anti-species antibodies and GNP was applied and dried on a glass fiber membrane. Antibodies against OTA were added to the sample in a free state as part of the dilution buffer. The detectable complex formed during ICA includes OTA-BSA, antibodies against OTA, anti-species antibodies, and GNP. The presence of OTA in the sample interferes with the formation of this complex, leading to a decrease in the intensity of staining up to complete disappearance.

Due to pre-incubation of the antigen with antibodies during the dilution stage, the duration of their interaction increases, which increases the sensitivity of the assay.In addition, production of the test strips is simplified and it becomes possible to quickly replace specific antibodies and optimize their concentration.

Table 1. Characteristics of two types of ICA for OTA

Parameter	Traditional Format	Indirect Labeling Format
Maximum staining intensity, rel. units	20.2	14.6
Instrumental limit of OTA detection, ng/mL	10	0.06
Visual limit of OTA detection, ng/mL	125	0.5
Duration of assay, min	15	20

Table 2. Completeness of OTA detection in corn extracts by the developed ICA

Introduced OTA, ng/mL	Found OTA, ng/mL	Degree of detection, %
1	0.95	95
0.5	0.51	102
0.25	0.24	96
0.125	0.142	114

Optimization of Immunoreagent Concentrations

To ensure that the ICA was highly sensitive, we varied the concentration of immunoanalytical reagents and compared the marker binding in the system at different concentrations of specific antibodies (from 1000 to 8 ng/mL). Based on the results (Fig. 3), we chose a concentration of 125 ng/mL as it produces the necessary staining intensity and does not lead to overexpenditure of antibodies, which would prevent sensitive competitive detection.

ICA with indirect labeling was carried out for the optimal concentration of antibodies against OTA as well as for the concentrations of OTA-BSA conjugate (1 mg/mL) and anti-species antibodies (A_{520} = 3) established in analogous experiments.

Characterization of ICA with Indirect Labeling

The chosen concentrations of immunoreagents were used to test a series of OTA solutions at concentrations of 0.01–1 ng/mL. Figure 4 shows the results obtained in this experiment when the incubation time of a sample and specific antibodies were varied.

In ICA without pre-incubation (Fig. 4, lower row), label binding was not completely inhibited, hindering visual evaluation of the results. Pre-incubation for 3 min is optimal as it enables maximum sensitivity but does not significantly extend the total duration of ICA.

Table 1 presents the characteristics of the developed ICA with indirect labeling and the traditional ICA, which was also implemented under optimal conditions. As shown, the transition to indirect labeling shifted the detection limit of the assay by almost two orders of magnitude for both visual and instrumental registration. However, the matrix of the tested samples may have had a significant impact on the assay results. Therefore, for the final characterization, the proposed ICA format was tested on corn kernel extract.

Verification of the New System using Plant Extracts

Complete OTA isolation from plant matrices can be achieved by using an extraction solution containing 70% methanol. However, high concentrations of organic solvents denature antibodies and inhibit immunoassays [17, 18]. Therefore, samples need to be diluted before ICA. Comparative analysis showed that the maximum methanol content after dilution that does not decrease marker binding in ELISA is 20%. This corresponds to the optimum value we established for ICA for other mycotoxins [19, 20]. Therefore, the initial extract was diluted 3.5 times with the buffer containing antibodies against OTA at the desired concentration. The calibration curve obtained for the ICA for OTA in real samples is shown in Fig. 5. The OTA detection limit with photometric registration is 0.12 ng/mL (i.e., the matrix was not observed to have a negative effect on the analytical characteristics of the assay system). This limit corresponds to the initial samples with an OTA content of 2.4 ng/g, which is lower than the regulatory threshold limit values of contamination. Experiments using the"introduced-found" scheme showed a high level of analyte detection (95–114%) (Table 2). Thus, the developed assay system satisfies the requirements of

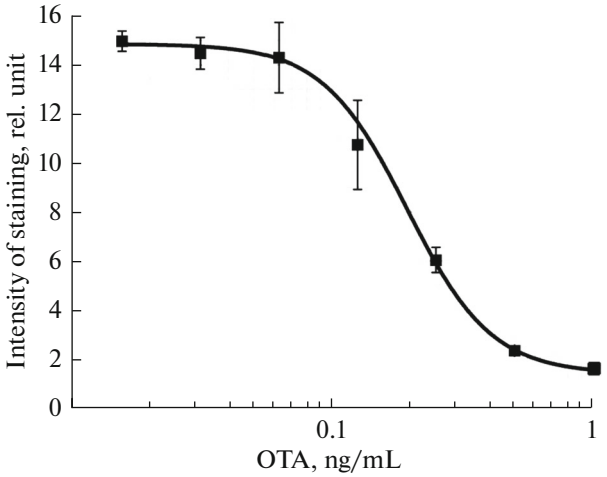

Fig. 5. Calibration curve of ICA for OTA in real samples (corn kernel extracts).

practice and appears to be a promising method for controlling the contamination of agricultural products.

CONCLUSION

A highly sensitive immunochromatographic assay system was developed to detect ochratoxin A. Transition to indirect labeling of antibodies was shown to reduce the detection limit for the analyte by two orders of magnitude. The proposed assay system is both rapid and sufficiently sensitive, making it beneficial for practical applications.

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