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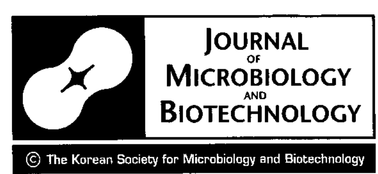

Development of Immunochromatography Strip-Test Using Nanocolloidal Gold-Antibody Probe for the Rapid Detection of Aflatoxin B1 in Grain and Feed Samples

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Abstract An immunochromatography (ICG) strip test using a nanocolloidal gold-antibody probe was developed and optimized for the rapid detection of aflatoxin B1 (AFB1). A monoclonal antibody specific to AFB1 was produced from the cloned hybridoma cell (AF78), coupled with nanocolloidal gold, and distributed on the conjugate pad of the ICG strip test. The visual detection limit of the ICG strip test was 0.5 ng/ml, and this method showed a cross-reaction to aflatoxin B2, G1, and G2. In total, 172 grain and feed samples were collected and analyzed by both the ICG strip test and HPLC. The results of the ICG strip test showed a good agreement with those obtained by HPLC. These results indicated that the ICG strip test has a potential use as a rapid and cost-effective screening tool for the determination of AFB1 in real samples and could be applied to the preliminary screening of mycotoxin in food and agricultural products, generating results within 15 min without complicated steps.

Keywords: Aflatoxin B1, mycotoxins, nanocolloidal gold, monoclonal antibody, immunochromatography strip test

Aflatoxins are toxic and carcinogenic secondary metabolites produced by *Aspergillus flavus* and *A. parasiticus*. Aflatoxins cause significant health and economic problems in the U.S.A. and many countries, because they have been frequently detected in food and agricultural commodities [1, 2]. They are listed as group I carcinogens by the International Agency for Research on Cancer (IARC) [15]. Aflatoxin B1 (AFB1) is the most commonly occurring toxic compound. AFB1 commonly contaminates agricultural commodities, such as grains, peanuts, corn, and feedstuffs. Regulations of aflatoxins have been set in many countries, although the maximum tolerated levels differ greatly among countries. The current maximum levels set by the European Union are 2 μg/kg for AFB1 and 4 μg/kg for total aflatoxins in groundnuts, nuts, dried fruits, and cereals [7], whereas Korean maximum levels are 10 μg/kg and 50 μg/kg for AFB1 in food and feed, respectively [16].

Many methods have already been proposed and reviewed for AFB1 determination in food and agricultural products [20, 39]. Current analysis of AFB1 is performed by thin-layer chromatography (TLC) [37], high-pressure liquid chromatography (HPLC) [3, 14], and immunoassays [19, 21, 23, 28, 34]. TLC is a relatively economical method of aflatoxin detection, with little equipment required, and still used broadly in developing countries. Although this method is simple and easy to perform, the sensitivity of TLC is low. HPLC is widely accepted as an official method for aflatoxin determination. However, this method is either expensive or time-consuming owing to the complication of sample preparation and preconcentration before determination [9]. In addition, both TLC and HPLC are unsuitable for the routine screening of large sample numbers. Immunoassays, such as enzyme-linked immunosorbent assay (ELISA), fluorescence polarization immunoassay (FPIA), and immunosensor [17, 22], provide a simple and economical alternative to instrumental methods for analysis. Moreover, immunoassays are sensitive and easy to perform [34]. In fact, for screening large number of samples, immunoassays are suitable tools [19, 21, 23, 28]. Specifically, ELISA in the surveillance of aflatoxins is becoming more widespread because of the sensitivity, specificity, rapidity, simplicity, and cost-effectiveness of the method. However, ELISA often requires long reaction times and involves multiple

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incubation and washing steps [29]. The detection of aflatoxins without several incubation and washing steps could be performed by FPIA [26, 27] or immunosensors [30, 33]. However, the utilization of these immunoassays has been confined to laboratories equipped with tools and special devices for the analysis.

There has been an increasing demand for monitoring of aflatoxins in developing regions such as South-East Asia, Africa, and the Middle East, where aflatoxins contamination is rampant. They are exporters of food and agricultural products, which can suffer from aflatoxins contamination. With the improvement of life standard, people demand more attention to food safety. There is an urgent need to develop a one-step assay for mycotoxin residues. Thus, many scientists attempted to develop a simple, rapid, and accurate method for the detection of aflatoxins in food and agricultural products. The convenience and speed of the test have been achieved by a novel concept of immunochromatography (ICG). The ICG technique, often called lateral-flow assay, is based on immunochromatographic procedures that utilize antigen and antibody properties for the rapid detection of an analyte [24, 35, 36, 38], thereby combining several benefits, including a user-friendly format, short assay time, long-term stability over a wide range of climates, and cost-effectiveness. These characteristics make it ideally suited for screening large numbers of samples by unskilled analysts [5]. This paper reports the development of a rapid, simple, and qualitative ICG strip test for AFB1 detection, and application of the method to the analysis of spiked and naturally contaminated grain and feed samples.

Fig. 1. Chemical structures of AFB1 (**A**), AFB1-oxime (**B**), and AFB1-protein conjugate (**C**).

Materials and Methods

Chemicals and Materials

Aflatoxins, other related mycotoxins, bovine serum albumin (BSA), ovalbumin (OVA), tetrachloroauric acid, complete and incomplete Freund's adjuvant, sodium citrate, and anti-mouse IgG were purchased from Sigma (St. Louis, MO, U.S.A.). Protein G agarose was purchased from Bioprogen (Daejeon, South Korea). Nitrocellulose membrane, sample pad, conjugate pad, and absorbent pad were obtained from Millipore (Bedford, MA, U.S.A.). Semi-rigid polyethylene sheets were purchased from a local market.

AFB1 derivative and protein conjugates with BSA and OVA were prepared in our laboratory according to the method previously described [6, 18]. AFB1-BSA conjugate was used as the immunogen and capture reagent on the test line of the ICG strip, and AFB1-OVA conjugate was used as the coating antigen in indirect ELISA for the determination of antisera titration. The chemical structures of AFB1, derivative, and AFB1-protein conjugate are shown in Fig. 1.

All standard solutions for aflatoxins and other mycotoxins were prepared by dilution of stock solutions of these compounds (1 mg/ml in methanol). All chemicals and organic solvents used were of analytical reagent grade.

Production of Monoclonal Antibody

Monoclonal antibody (MAb) to AFB1 was produced in our laboratory by the standard procedure [12] using AFB1-BSA conjugate as the immunogen. Five 7-week-old BALB/c mice were immunized with 100 μg of AFB1-BSA conjugate in 0.1 ml sterilized 0.05 M phosphate-buffered saline (PBS, pH 7.4), which was emulsified with an equal volume of Freund's complete adjuvant. Boost injections were given 2 and 4 weeks later. Ten days later, antisera were collected from the caudal vein of each mouse and titers of antisera were determined by indirect ELISA using AFB1-OVA as coating antigen. Three days before cell fusion, the mice that produced antisera with high titers were given another intraperitoneal boost injection without adjuvant. For cell fusion, suspensions of 3.5×10^8 spleen cells from immunized mice were fused with 4.3×10^8 murine myeloma cells (P3-X63-Ag8.653) using PEG 4000. After HAT selection, the supernatants of fused cells were assayed by indirect ELISA. ELISA-positive fused cells were cloned by the limiting dilution method. Cloned hybridoma cells (1.0×10^7 cell in 0.2 ml of PBS) were intraperitoneally injected into BALB/c mice, pretreated with an intraperitoneal injection of 0.5 ml pristane, and grown as ascite tumors. The immunoglobulin fraction was prepared from the ascites fluids by precipitation with saturated ammonium sulfate

followed by affinity chromatography on a protein G column. The protein concentration of the purified MAb was determined with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.). The isotype of the cloned MAb was determined with a mouse monoclonal antibody isotyping kit (Roche Applied Science, Switzerland) according to the instructions.

Synthesis of Nanocolloidal Gold-MAb Probe

Nanocolloidal gold particles (diameter 40 nm) were produced in our laboratory according to the method of Frens [11], and gold particles were labeled with MAb by the method of Roth [31, 32]. Before conjugation of the MAb and nanocolloidal gold, the minimum amount of MAb needed for stabilization of the nanocolloidal gold solution was determined. Briefly, 0.1 mg of lyophilized MAb was dissolved in 1 ml of 2 mM borax and the nanocolloidal gold solution was adjusted to pH 8.5 with 0.1 M K_2CO_3. One ml of nanocolloidal gold was distributed into each of a series of 1.5-ml tubes. MAb solution (0–150 μl) was added to each tube. The tubes were shaken for 1 min and then incubated for 5 min at room temperature. One-hundred μl of 10% (w/v) NaCl was added to each tube and mixed for 1 min. The minimum amount of MAb was evaluated by color change from reddish to blue. If a tube contained the minimum amount of the antibody, the color of the nanocolloidal gold solution did not change from reddish to blue. For conjugation, 30 ml of nanocolloidal gold solution was mixed dropwise with 3 ml of MAb solution (0.1 mg/ml) in a tube under rapid stirring. After 1 h, 3.6 ml of 10% (w/v) BSA was added for blocking of the residual surface of the nanocolloidal gold particles and the mixture was incubated for 1 h at room temperature. The mixture was then centrifuged for 15 min at 10,000 rpm and the supernatant was discarded. The pellets were resuspended in 2 mM borate buffer (pH 7.2). The centrifugation was repeated twice and final pellets were resuspended with 3 ml of 2 mM borate buffer (pH 7.2) containing 1% (w/v) BSA, 1% (w/v) sucrose, and 0.05% (w/v) sodium azide. The nanocolloidal gold-antibody probes were stored at 4°C before use.

Development of ICG Strip Test

An ICG strip test consisted of three pads (sample, conjugate, and absorbent pad) and one nitrocellulose membrane with test and control lines. A schematic diagram of the ICG strip is shown in Fig. 2. The sample pads and absorbent pads were treated according to the method described in a previous paper by the current authors [35]. Five μl of the nanocolloidal gold-MAb probe (absorbance at 540 nm was 1.5) was applied to the conjugate pad and allowed to dry for 30 min at 37°C. The test and control lines on the nitrocellulose membrane were treated with 3 μl of AFB1-BSA conjugate (0.5 mg/ml in PBS) and 3 μl of goat anti-mouse IgG (1.0 mg/ml in PBS), and allowed to dry for 30 min at 37°C. The treated pads and the membranes were all attached to a semi-rigid polyethylene sheet. A positive test showed only one red line in the control line, whereas a negative test showed two red lines in the test and control lines. The ICG strip test was incorrect if there was no red line in the control line (Fig. 2)

HPLC

Rice, barley, and feed samples were extracted with extraction solution (methanol:water:n-hexane=15:10:10) and the

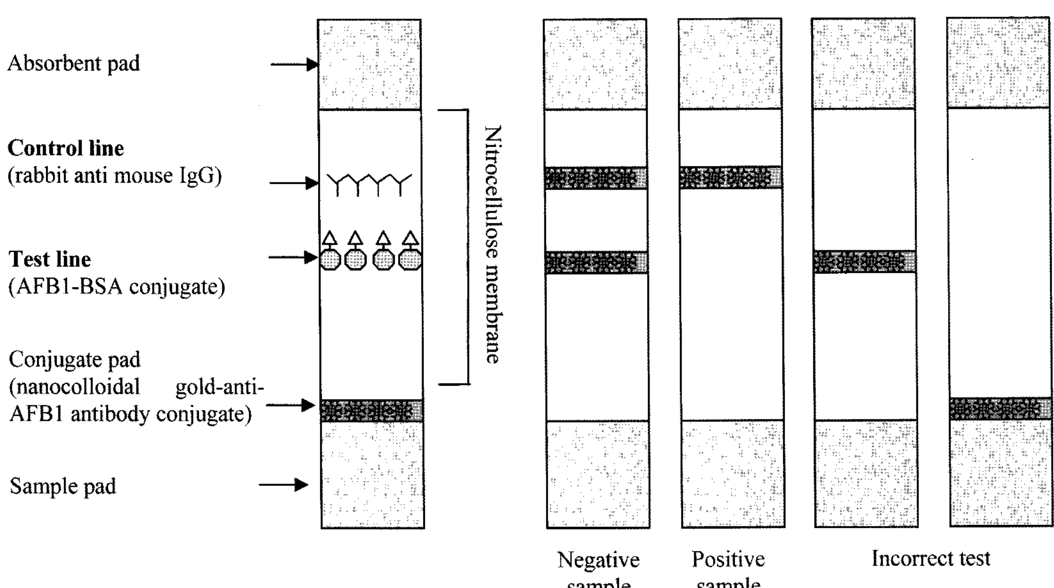

Fig. 2. Schematic diagram of the ICG strip test.

extracts were purified using a chromatograph column (22× 330 nm) with silica gel (Merck, Darmstadt, Germany) as resin and chloroform. The method of instrumental analysis for HPLC was the same as described in the paper by Han *et al.* [13].

Sample Collection and Analysis

Sixty-four rice samples and 43 barley samples were taken from traditional markets in Gyeonggi, Chungnam, Chungbuk, Gyeongnam, Gyeongbuk, Jeonnam, Jeonbuk, and Gangwon provinces, South Korea. Sixty-five feed samples were kindly provided by Professor Dong-Ho Bae from Konkuk University (Seoul, South Korea). To evaluate the ICG test strip, one gram of dried ground samples, which are AFB1 negative by HPLC, was spiked with AFB1 at different concentrations (0, 5, 10, 20, 50, and 100 ng/ml) and then left overnight at room temperature. The spiked samples were extracted with 5 ml of 60% (v/v) methanol containing 4% (w/v) NaCl for 30 min at room temperature with shaking at intervals. The extracts were centrifuged at 2,000 rpm for 10 min. To decrease matrix interferences and methanol concentration, the grain and feed extracts were diluted 2-fold and 4-fold with PBS. The diluted sample extracts (200 µl) were analyzed in triplicate by ICG strip test. Blank and naturally contaminated samples were prepared as described above but not spiked with AFB1. Monitoring of AFB1 for the collected grain and feed samples was performed by both the ICG strip test and HPLC.

RESULTS AND DISCUSSION

Characterization of MAb to AFB1

The molecular weight of AFB1 is 312, which is too small to be used as an immunogen by itself. Therefore, the synthesis of hapten and conjugation to carrier protein are necessary and important in the preparation of desirable immunoreagents. In order to overcome the limit of small-molecule recognition in an immune response, AFB1 was derived to AFB1-oxime and this hapten was conjugated with BSA and used as the immunogen instead of AFB1 itself (Fig. 1). However, immunized mice with AFB1-BSA might induce many kinds of antibodies that recognized not only AFB1 but also BSA. Antisera from immunized mice showed a high titer in indirect ELISA and the mice were all used for cell fusion. Five cloned hybridoma cells (AF29, AF34, AF58, AF62, and AF78) that produced MAbs to AFB1 were generated after the cell fusion and cloning steps. Their culture fluids were evaluated by indirect competitive ELISA using AFB1-OVA conjugate as the coating antigen. Four culture media from cloned hybridoma cells (AF34, AF58, AF62, and AF78) showed a good competition between free AFB1 and AFB1-OVA. This result means that MAbs produced from the four hybridoma cells truly react to AFB1 without nonspecific binding to BSA. Specifically, the culture fluid of AF78 hybridoma cells showed the highest sensitivity (IC_{50}=1.0 ng/ml) in indirect competitive ELISA. Thus, we selected and expanded AF78 hybridoma cells for MAb mass production and purification. The protein concentration of the MAb was determined to be 1.0 mg/ml by the Bio-Rad protein assay kit. The isotype of the MAb was determined using an isotyping kit and found to be IgG_1 subclass with a κ-type light chain. The cross-reactivity of MAb to aflatoxins and other mycotoxins is shown in Table 1. The MAb produced in this study showed 26%, 61%, and 24% cross-reactivities toward aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2), respectively. These indicated that AF78 MAb possesses a good affinity to total aflatoxins and could be applied to develop an immunoassay for total aflatoxins detection.

Characterization of ICG Strip Test

Nanocolloidal gold is commonly used as an immunospecific probe for immunocytochemistry and immunoblotting [10, 25]. Many scientists recognized that nanocolloidal gold particles could be applied in immunoassays, biosensors, gene therapy, and DNA computation [8]. In this study, nanocolloidal gold with a diameter of 40 nm was produced in our laboratory using the method of Frens [11], because the 40 nm gold particles offered maximum visibility owing to the least steric hindrance in the case of IgG conjugation [4], and conjugated with MAb for use as a marker. Spherical gold particles formed when 1 ml of sodium citrate was added to 100 ml of 0.01% (w/v) tetrachloroauric acid. Before conjugation of the nanocolloidal gold and MAb, a minimum amount of MAb for the stabilization of nanocolloidal gold should be checked. The minimum amount of MAb was determined by adding NaCl to nanocolloidal gold particles containing different amounts of MAb [35]. In our study, although 9 µg of MAb was confirmed to be the minimum amount for stabilization of nanocolloidal gold, we selected 10 µg of MAb for the conjugation because 9 µg of MAb often caused coagulation of gold particles during the conjugation.

Since the main objective of the ICG strip test was the qualitative detection of AFB1, it was important that the color intensity of the test line was strong enough to be seen and to enable a clear distinction between negative and positive tests. Therefore, to develop a sensitive ICG strip test, the optimal immobilization amount of AFB1-BSA conjugate applied to the test line and optimal amount of nanocolloidal gold-MAb sprayed onto the conjugate pad were determined. A negative test developed a clear red line on the test line within the shortest time, and the color intensity on the test line between negative and positive tests could be easily distinguished by the naked eye (Fig. 2). The optimal conditions for ICG strip test were as follows: 1.5 µg of

Table 1. Cross-reactivity of AF78 MAb to aflatoxins and other mycotoxins by indirect competitive ELISA.

Mycotoxins	Structure	IC_{50} (ng/ml)	Cross-reactivity (%)
Aflatoxin B1		1.0	100
Aflatoxin B2		3.9	26
Aflatoxin G1		1.7	61
Aflatoxin G2		4.4	24
Ochratoxin A		ni[b]	0
Citrinin		ni	0
Patulin		ni	0
Zearalenone		ni	0
T-2 toxin		ni	0

[a]No inhibition.

AFB1-BSA conjugate was treated in the test line on the membrane and 5 μl of nanocolloidal gold-MAb probe (absorbance at 540 nm was 1.5) was sprayed on the conjugate pad. Two-hundred μl of standard or sample was applied to the sample pad and then allowed to migrate up the membrane. The results were evaluated visually by eye within 15 min after starting the reaction. A series of dilutions of AFB1 standard (0–10 ng/ml) were tested by the ICG strip test and the result is shown in Fig. 3. Whereas a weak signal in the test line was shown at 0.1 ng/ml of AFB1, no signal in the test line was observed at >0.5 ng/ml of AFB1. Thus, we selected 0.5 ng/ml as the detection limit of the ICG strip test by eye for the assurance of the results.

The specificity of the ICG strip test was evaluated with aflatoxins and other mycotoxins. As shown in Fig. 4, two

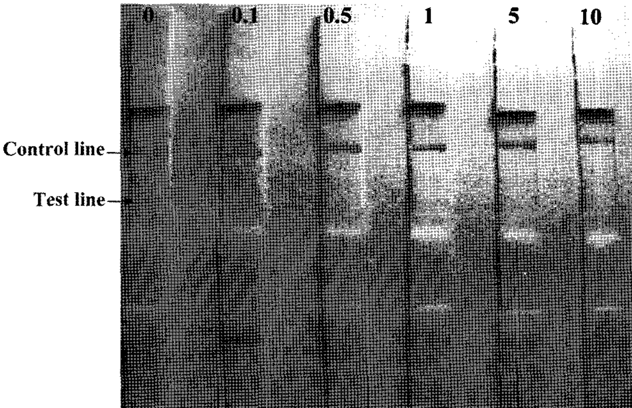

Fig. 3. Detection limit of the ICG strip test for the detection of AFB1.
The tests were run four times at room temperature using 10% MeOH/PBS spiked with various AFB1 standards. The labels (0, 0.1, 0.5, 1, 5, and 10) indicate the concentrations of AFB1 (ng/ml).

red lines were clearly observed in the test and control lines when other mycotoxins were applied to the ICG strip test. This means that the ICG strip test developed in this study does not react to other mycotoxins. The cross-reactivity for aflatoxins such as AFB2, AFG1, and AFG2 was observed. As a result, we deduced that the ICG strip test could be applied to detect not only AFB1 but also total aflatoxins.

Recovery Studies

Because of the low solubility of AFB1 in aqueous solution, such as water or PBS, methanol is generally used to extract AFB1 from food and agricultural products. However, methanol tends to dissolve protein and lipid, which could interfere with AFB1 determination. To reduce matrix interferences, two common approaches have been used. The first approach is sample cleanup, which is laborious and time-consuming and may affect assay reproducibility and recovery. The second approach is dilution of the extract. One of the major advantages of immunoassay techniques is their simplicity, so the second approach was used in this study. In our study, rice, barley, and feed samples were extracted with 60% (v/v) MeOH containing 4% (w/v) NaCl. After extraction, the dilution time of the extracts was determined because the extracts contained many obstacles, which could interfere with binding between the AFB1 and nanocolloidal gold-MAb, and the immunoreagents (nanocolloidal gold-MAb probe, AFB1-BSA, and anti-mouse IgG) used in the ICG strip test are unstable in 60% (v/v) methanol. When the original extracts were applied to the ICG strip test, no signals in the test and control lines were observed, whereas after 2-fold and 4-fold dilution with PBS for grain and feed extracts that did not contain AFB1, the same results with 10% methanol without AFB1were obtained by the ICG strip test (data not shown). Thus, we selected 2-fold and 4-fold dilutions as

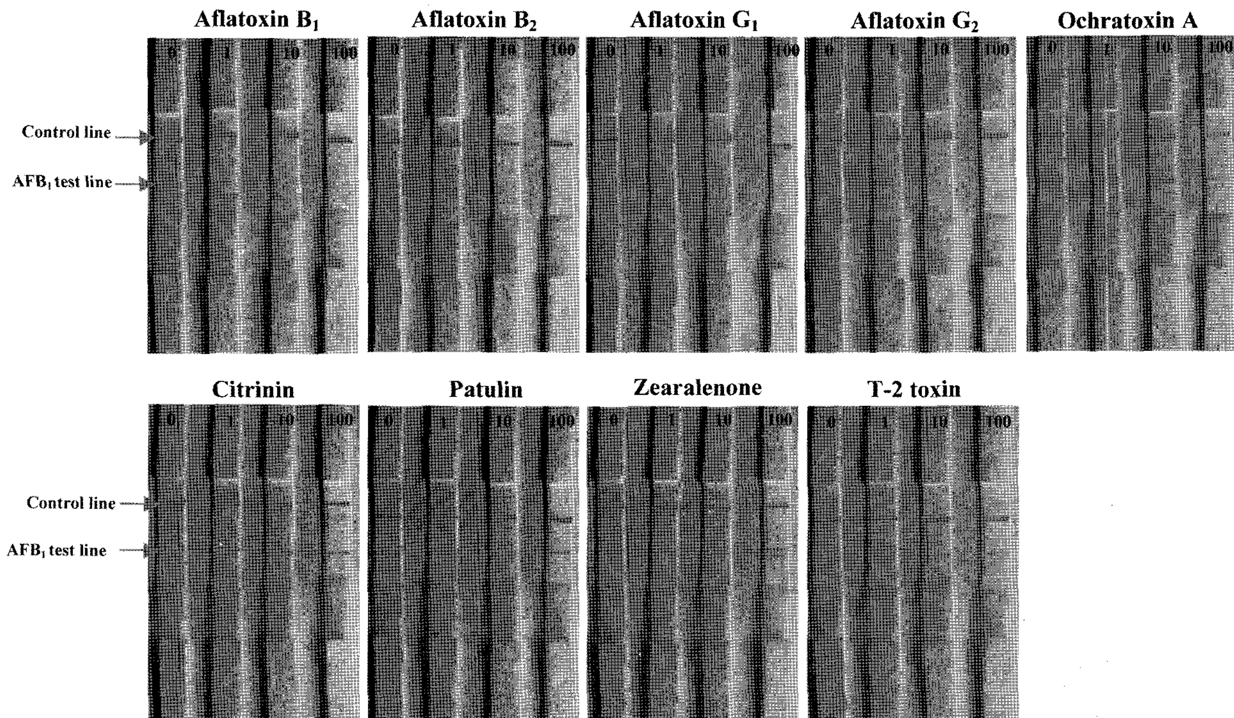

Fig. 4. Cross-reactivities to aflatoxins and other mycotoxins by the ICG strip test.
The tests were run four times at room temperature using 10% MeOH/PBS spiked with various mycotoxin standards. The labels (0, 1, 10, and 100) indicate the concentration of mycotoxins (ng/ml).

Table 2. Analysis of rice, barley, and feed samples spiked with AFB1 by the ICG strip test.

Sample	Spiked AFB1 concentration (μg/kg)	ICG strip	
		Test line	Control line
Rice (n=3)	0	+[a]	+
	5	±[b]	+
	10	–[c]	+
	20	–	+
	50	–	+
	100	–	+
Barley (n=3)	0	+	+
	5	±	+
	10	–	+
	20	–	+
	50	–	+
	100	–	+
Feed (n=3)	0	+	+
	5	±	+
	10	±	+
	20	–	+
	50	–	+
	100	–	+

[a]An obvious red band was observed.
[b]A faint band was observed.
[c]No band was observed.

the minimum dilution times for grain and feed samples because increasing the dilution will cause decrease of the AFB1 concentration in the samples. For recovery studies, grain and feed samples, uncontaminated by AFB1, were spiked with different AFB1 concentrations (5, 10, 20, 50, 100 μg/kg). The AFB1-spiked samples were extracted as described above and analyzed by the ICG strip test. As shown in Table 2, weak red lines on the test line were observed when the spiked grain with <5 μg/kg AFB1 and feed with <10 μg/kg AFB1 were applied to the ICG strip test, whereas no red lines on the test line were shown in analysis of the spiked grain with >10 μg/kg AFB1 and feed with >20 μg/kg AFB1. In addition, the obvious red lines on the test line were shown in application of grain and feed samples without spiking of AFB1. After sample preparation, the final AFB1 concentration of the spiked grain with >10 μg/kg AFB1 and feed with >20 μg/kg AFB1 was all >1 ppb. Fortunately, these levels could be detected by the ICG strip test. We are certain that the ICG strip test has a potential use as a rapid and cost-effective screening tool for the determination of AFB1 in real samples.

Sample Analysis

Rice (64 samples), barley (43 samples), and feed (65 samples) were analyzed by both the ICG strip test and HPLC (Table 3). In the HPLC analysis, 7 barley samples and most of the feed samples were found to be AFB1 contaminated. However, although 5 feed samples (AFB1 level: >1 μg/kg) were confirmed as AFB1-positive by the ICG strip test, all grain samples and most feed samples (AFB1 levels: <1 μg/kg AFB1) were AFB1-negative. The results of the ICG strip test for the feeds, which were contaminated with >1 μg/kg of AFB1, showed a good agreement with those obtained by HPLC. One (Jn2) feed sample showed the highest AFB1 level (15.58 μg/kg), but AFB1 levels of all positive samples by HPLC were lower than Korean guidelines for AFB1 (10 μg/kg AFB1 for food and grain, and 50 μg/kg AFB1 for feed).

An immunochromatographic assay using a polyclonal antibody-gold probe has been reported for the detection of AFB1 [38], but the sensitivity of this method (detection limit: 2.5 ng/ml) was lower than the ICG strip test developed in this study. A commercial kit based on the immunochromatographic assay has been developed and applied to determine AFB1 in food and agricultural products. However, the detection limit of the commercial kit is 20 ng/ml for AFB1 and this sensitivity is unsuitable for Korean guidelines [16]. Therefore, a more sensitive method is needed for AFB1 detection among the Korean scientists, and a more sensitive ICG strip test suited for Korea guidelines was developed in this study.

In conclusion, the ICG strip test developed in this study showed a high sensitivity and the detection limit of the ICG strip was 0.5 ng/ml. For enhancement of sensitivity, several scientists have used a strip reader. If our method will be measured by the strip reader, the enhancement of sensitivity will be obtained. The ICG strip test is easy to perform and the results could be obtained within 15 min without the need of expensive equipment, washing, and/or separation step. Comparative analyses of naturally contaminated grain and feed samples performed by both the ICG strip test and HPLC showed a good agreement (Table 3). With respect to

Table 3. Results of AFB1 analysis in rice, barley, and feed samples by the ICG strip test and HPLC.

Samples	Number of tested samples	Qualitative results by ICG strip test	Quantitative results by HPLC	
		Number of positive samples (sample label)	AFB1 level (<1 μg/kg)	AFB1 level (≥1 μg/kg)
Rice	64	0	0	0
Barley	43	0	7	0
Feed	65	5 (Ch1, Ch7, Jn2, Jn4, Gi1)	MFS[a]	5 (Ch1, Ch7, Jn2, Jn4, Gi1)

[a]Most feed samples.

its overall speed and simplicity, the assay is superior to other immunoassays, such as the fluorescence polarization immunoassay and enzyme-linked immunosorbent assay. These results in this study indicated that the ICG strip test was sufficiently sensitive and accurate to be useful for rapid screening of AFB1 in various agricultural products and food samples. Moreover, the ICG strip test could detect total aflatoxins because the MAb used in the ICG strip has a high cross-reaction to AFB2, AFG1, and AFG2. Finally, the ICG strip test has potential as a rapid and cost-effective screening tool for AFB1 determination in agricultural and food samples and could be applied to the preliminary screening of mycotoxins in agricultural and food samples.

Acknowledgments

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