Determination of Aflatoxin B1 in Rice, Barley, and Feed by Non-instrumental Immunochromatographic Strip-test and High Sensitive ELISA

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Abstract A non-instrumental immunochromatographic (ICG) strip-test and direct competitive enzyme-linked immunosorbent assay (DC-ELISA) for aflatoxin B1 (AFB1) determination were developed and optimized. The detection limits of ICG strip-test and DC-ELISA were 0.5 and 0.004 ng/mL, respectively, and these methods possessed a cross-reaction to aflatoxins. The results of spiked samples by both methods were coincided with the amount spiked AFB1 and the comparative analyses of 172 real samples by 2 immunoassays and high performance liquid chromatography (HPLC) showed a good agreement. Especially, the ICG strip-test is easier to perform and quicker, but less sensitivity than DC-ELISA. Both methods could analyze a high sample throughput with short time, but the sample throughput of ICG strip-test was better. Therefore, the ICG strip-test can be used as a simple, easy, non-instrumental, and fast screening technique for AFB1 determination.

Keywords: aflatoxin B1, mycotoxin, non-instrumental assay, immunochromatographic strip-test (ICG), enzyme-linked immunosorbent assay (ELISA)

Introduction

Aflatoxins produced from several *Aspergillus* spp., including *A. parasiticus* and *A. flavus* are very toxic and carcinogenic to animal and human. Because these mycotoxins commonly contaminate in food and agricultural commodities, they cause significant health and economic problems in many countries (1). Especially, aflatoxin B1 (AFB1) is listed as group I carcinogens by the International Agency for Research on Cancer (IARC) (2) and the most significantly occurring and toxic compound. Many countries have set severe regulatory demands on the level of aflatoxins permitted in foods and agricultural commodities. The current maximum level set by the European Union is 2 µg/kg for AFB1 and 4 µg/kg for total aflatoxins in groundnuts, nuts, dried fruits, and cereals (3), whereas the Korean government has the maximum levels for AFB1 (10 µg/kg for grain and food, and 50 µg/kg for feed) (4,5). Actually, AFB1 has been mainly detected in peanut, grain, and feed because fungi which could produce AFB1 can grow in commodities before harvest, during the time between harvesting and drying, and in storage.

To determine AFB1 in food and agricultural products, many methods have been proposed since the discovery of AFB1 (6,7). Current analysis of AFB1 is carried out by thin layer chromatography (TLC) (8), high performance liquid chromatography (HPLC) (9,10), and immunoassays (11-14). However, the conventional methods, such as TLC

and HPLC require an extraction, complicated clean-up, enrichment step prior to determination, and sophisticated technical equipment. Therefore, they are mostly laborious, time-consuming, and unsuitable for the routine screening of large sample numbers (15). Therefore, the development of rapid method for AFB1 is needed among many scientists.

Immunoassays provide a simple and economical alternative to instrumental methods for AFB1 analysis and are being used increasingly for screening of AFB1 in food and agricultural commodities. Over the last 20 years, the importance and application of immunoassays, especially enzyme-linked immunosorbent assay (ELISA), have grown significantly. ELISA is not only suitable tools for quick and sensitive analysis with high sample throughput (12,13) but also cost-effective, fast, and need a small sample volume for analysis (16). However, the utilization of this method has been often confined to laboratories equipped with tools and devices for analysis (17,18). In recent years, an immunochromatographic (ICG) strip-test using colloidal gold-antibody probe has been reported for the detection of mycotoxins (19,20), antibiotics (21), pesticides (22,23), and pathogenic bacteria (24) in food and agricultural products. This method provides a rapid detection for mycotoxins without special equipments and several benefits, such as 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 on-site screening by untrained personnel (20). A commercial kit based on ICG assay has been developed and applied to determine AFB1 in food and agricultural products. However, the detection limit of the commercial kit is approximately 20 ng/mL of aflatoxin and this sensitivity is unsuitable for

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Korean guide line. Therefore, more sensitive ICG strip-test needed for AFB1 detection among the Korean scientists.

In this study, a non-instrumental ICG strip-test and high sensitive direct competitive (DC)-ELISA based on a monoclonal antibody (MAb) for detection of AFB1 was developed and the comparison of analytical parameters for 2 immunoassays was described. Additionally, the analytic results of 172 samples by both methods were also compared with those obtained by HPLC.

Materials and Methods

Materials and chemicals Nitrocellulose membrane, sample pad, conjugate pad, and absorbent pad were obtained from Millipore (Bedford, MA, USA). Semi-rigid polyethylene sheets were purchased from local market. Microtiter plates (96 wells) were obtained from Nalgen Nunc International (Rockilde, Denmark). Aflatoxins, other related mycotoxins, bovine serum albumin (BSA), ovalbumin (OVA), tetra-chloroauric acid, sodium citrate, complete and incomplete Freund's adjuvant, N,N-dicyclohexylcarbodiimide, salts, horseradish peroxidase (HRP), and goat anti-mouse IgG were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Protein G agarose for the purification of MAb was purchased from Bioprogen (Daejeon, Korea). All standard solutions for aflatoxins and other related 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.

Preparation of immunoreagents AFB1-BSA and AFB1-HRP conjugates were synthesized in our lab according to previously described (25). AFB1-BSA conjugate was used as an immunogen in immunization and capture reagent in ICG strip-test, respectively, and AFB1-HRP conjugate was used as a competitor to free AFB1 in DC-ELISA. MAb to AFB1 (AF78 MAb) was produced in our laboratory according to the standard procedure (26), has already been characterized by ELISA, and was found to be highly specific for AFB1.

In DC-ELISA, AFB1-HRP conjugate was generally used as a marker or tracer. However, for the ICG strip-test, a colloidal gold particle (diameter 40 nm) conjugated with an antibody or antigen was employed as a marker. A colloidal gold particle was produced according to the method of Frens (27) and it was coupled with MAb according to the method of Roth (28). Briefly, after boiling an aqueous solution of tetrachloroauric acid (100 mL of 0.01% $HAuCl_4 \cdot 3H_2O$), 1 mL of 1% sodium citrate was rapidly added. The mixture was strongly stirred while gently boiling until the color of this solution changed from purple to red. After cooling, 10 mL of colloidal gold solution was mixed with 1 mL of MAb solution (0.1 mg/ mL in 2 mM borax) in a tube under rapid stirring and incubated for 1 hr at room temperature (RT). Then 1.2 mL of 10% BSA was added to the mixture to block residual surface of the colloidal gold particles. After incubation for 1 hr at RT, the mixture was centrifuged for 15 min at 9,800×g and the supernatant was discarded. A pellet was resuspended in 2 mM borate buffer (pH 7.2) and centrifugation was repeated twice. The final pellet was resuspended with 1 mL of 2 mM borate buffer (pH 7.2)

containing 1% BSA, 1% sucrose, and 0.05% sodium azide and were stored at 4°C before use.

DC-ELISA The wells of microtiter plates were precoated overnight at 4°C with 100 μL of anti-mouse IgG [20 μg/ mL in phosphate buffer saline (PBS, pH 7.4)] and then washed with PBS containing 0.05% Tween 20 (PBST) by using a Nunc-Immuno Wash 8 microplate washer (Nalge Nunc International). The precoated wells were coated with 100 μL of MAb solution (0.5 μg/mL in PBS) for 1 hr at 37°C and then washed with PBST. Fifty μL of AFB1 standards (or samples) and 50 μL of AFB1-HRP solution (0.7 μg/mL in PBST) were added to the wells and incubated at RT for 20 min with shaking. The plates were washed and 100 μL of 0.1 M citrate buffer (pH 4.0) containing 0.025% 2,2'-azinobis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) and 0.03% H_2O_2 was added. After incubation for 20 min at 37°C, color development was stopped with 2 M H_2SO_4 (50 μL per well) and the absorbance was measured at 405 nm using a Bio-Rad model 550 microplate reader (Bio-Rad Laboratories, Richmond, CA, USA).

ICG strip-test The schematic diagram and illustrate of non-instrumental ICG strip-test for the detection of AFB1 was shown in Fig. 1. An ICG strip-test consisted of 3 pads, such as sample, conjugate, and absorbent pad, and one nitrocellulose membrane containing test and control zone. The conjugate pads were treated with the colloidal gold-MAb probe and dried at 37°C for 30 min. The test and control zones on nitrocellulose membrane were arranged with AFB1-BSA conjugate and goat anti-mouse IgG and dried at 37°C for 30 min. The sample pads were soaked with 50 mM borate buffer (pH 7.4) containing 1% BSA, 0.5% Tween 20, 5% sucrose, 5% dextrane, and 0.05% sodium azide and then dried for 1 hr at 60°C. An absorbent pad was used without treatment. The treated pads and the membranes were all attached to a semi-rigid polyethylene sheet.

Sample preparation Five g of the AFB1-free samples (rice, barley, and feed) confirmed by HPLC were immersed in 25 mL of 60% methanol containing 4% NaCl and shaken for 15 min at RT. After centrifugation at 2,500×g for 10 min, all extracts were filtered through filter paper. The grain (rice and barley) and feed extracts were diluted 2- and 4-fold with PBS to decrease matrix interferences and methanol concentration and used immediately in ICG strip-test. For DC-ELISA analysis, the extract was diluted 5-fold with PBST.

Analysis of AFB1 in artificially spiked and natural samples The spiked samples (0, 5, 10, 20, 50, and 100 μg/kg) were prepared by adding methanol containing different concentrations of AFB1 to 5 g of AFB1-free samples and allowed to dry overnight at RT. Blank samples were prepared without spiking. The spiked and blank samples were extracted and prepared as described above and analyzed in triplicate by ICG strip-test and DC-ELISA. A total of 172 samples (64 rice, 43 barley, and 45 feed samples) were taken from traditional markets in the 8 provinces (Gyeonggi, Chungnam, Chungbuk, Gyeongnam,
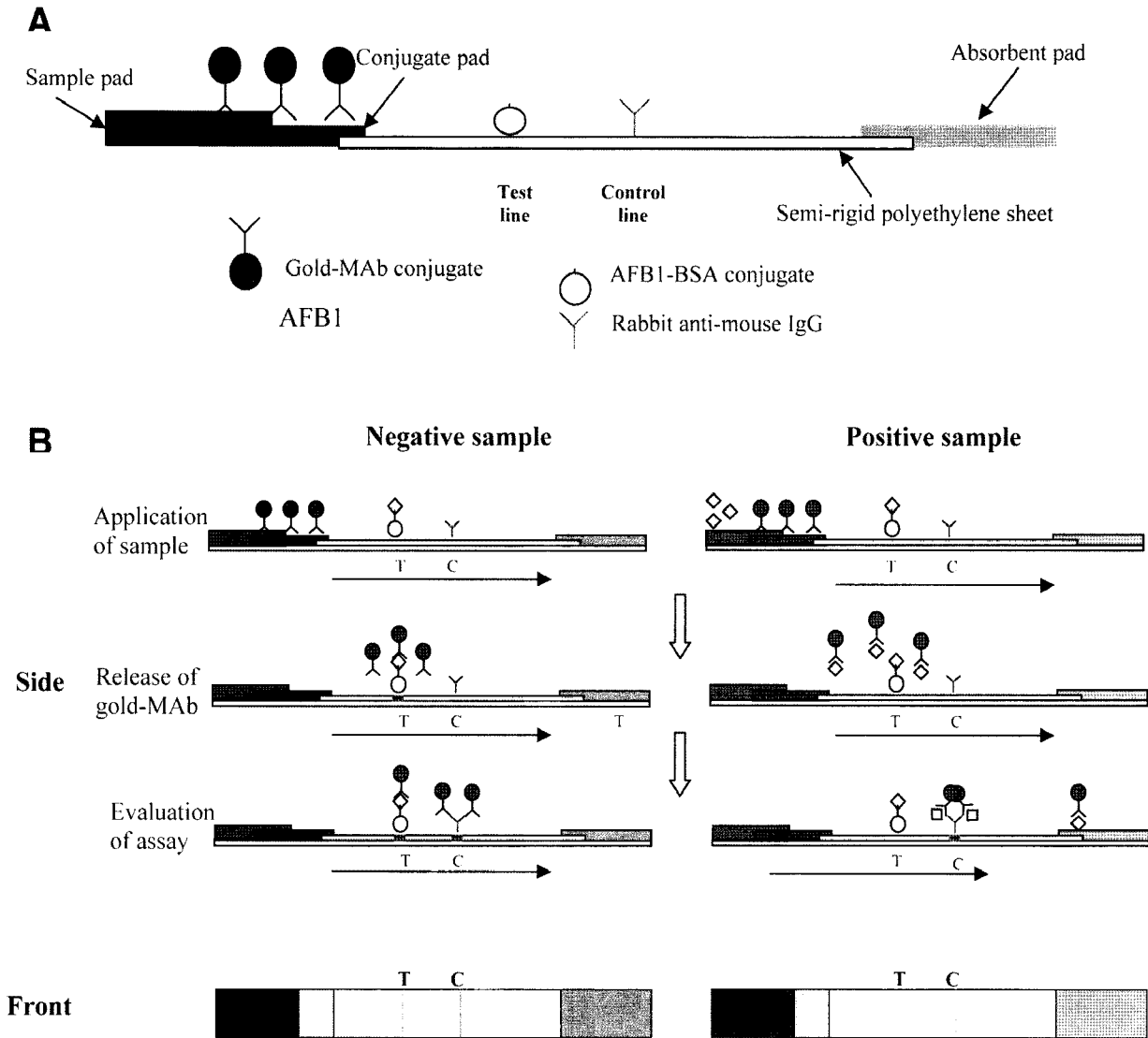
A

B

Fig. 1. Construction of ICG strip-test (A) and illustration of its results (B). The test is invalid if no line is appeared in the control line. C, control line; T, test line.

Gyeongbuk, Jeonnam, Jeonbuk, and Gangwon) of Korea and analyzed by DC-ELISA, ICG strip-test, and HPLC. For HPLC analysis, rice, barley, and feed samples were extracted with extraction solution (methanol : water : *n*-hexane = 15:10:10) and the extracts were purified using a chromatograph column (22×330 nm) with silica gel (Merck, Darmstadt, Germany) and chloroform. Method of instrumental analysis for HPLC was performed according to previously described (29).

Results and Discussion

Characterization of DC-ELISA To develop a sensitive DC-ELISA, the optimal concentrations of immunoreagents were established by titrating an AFB1-HRP conjugate against MAb coated on microplate well at different concentrations and determining the concentrations producing an optical density of 1-1.5 at 0 ng/mL of AFB1. The optimal combination of AFB1-HRP conjugate (0.7 μg/mL in PBST) and MAb (0.5 μg/mL in PBS) offered the best sensitivity. Figure 2 shows a typical standard curve of DC-

ELISA in PBS containing 10% methanol. The IC_{50} value was 0.01 ng/mL and the detection limit (10% inhibition) was 0.004 ng/mL. Cross-reactivity of DC-ELISA to aflatoxins and other mycotoxins was shown in Table 1. This assay exhibited 26, 31, and 23% cross-reactivity toward aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2), respectively, but no cross-reaction was observed with other mycotoxins, such as ochratoxin A, citrinin, patulin, deoxynivalenol, zearalenone, T-2 toxin. The sensitivity of the DC-ELISA developed is sufficient to detect AFB1 levels of Korean legal limit for food (10 μg/kg) and feed (50 μg/kg). Thus, we think that this method should be became accurately detectable method for AFB1 in natural samples.

Characterization of ICG strip-test Main advantages of ICG strip-test are a user format, on-site, and non-instrumental detection. Recently, the strip reader has been usually used to enhance sensitivity of ICG strip-test. With respect to on-site detection, the use of special equipments is unsuitable. To develop a high sensitive and non-instrumental ICG
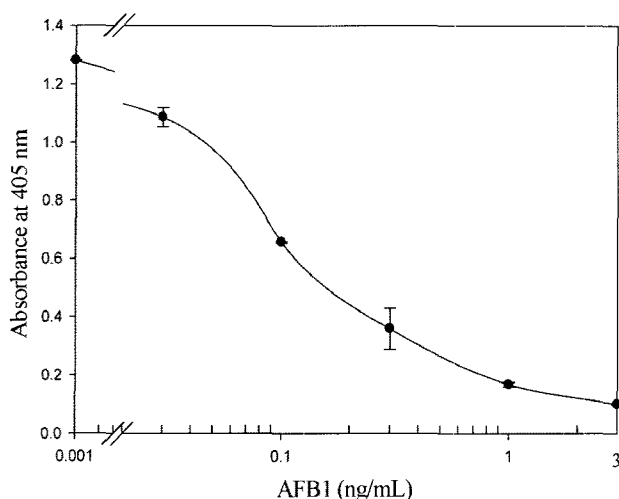
Fig. 2. DC-ELISA standard curve for AFB1 detection in PBS containing 10% methanol. Each point of the curve represents the mean±SD of n=4 assays on the same day.

Table 1. Cross-reactivity of MAb to aflatoxins and other mycotoxins by both DC-ELISA and ICG strip-test

Mycotoxins	Cross-reactivity[1]	
	DC-ELISA (%)	ICG strip-test
Aflatoxin B1	100	+ ($\geq$0.5 ng/mL AFB1)
Aflatoxin B2	26	+ ($\geq$3 ng/mL AFB1)
Aflatoxin G1	31	+ ($\geq$1 ng/mL AFB1)
Aflatoxin G2	23	+ ($\geq$3 ng/mL AFB1)
Ochratoxin A	0	-
Citrinin	0	-
Patulin	0	-
Zearalenone	0	-
Deoxynivalenol	0	-
T-2 toxin	0	-

[1]+, Positive, -, negative results in ICG strip-test; AFB1 levels showing positive results by ICG strip-test.

strip-test for AFB1, the antibody possessed high sensitivity and AFB1-protein conjugate are necessary. AF-78 MAb showed high sensitivity in the DC-ELISA and high affinity to AFB1-BSA conjugate in preliminary work, so we expect that high sensitive and non-instrumental ICG strip-test could be developed if these are used in development of ICG strip-test.

After conjugation of colloidal gold and MAb, the residuary active site of gold was blocked with 100 μL of 10% BSA, but it often causes a non-specific binding to immunoreagents (AFB1-protein conjugate and anti-mouse ICG) immobilized on membrane. Thus, the suitability of gold-MAb conjugate in ICG strip-test was evaluated and its result was shown in Fig. 3. The colloidal gold-BSA conjugate did not react to immunoreagents immobilized on the test and control zones, as there were no red lines on the nitrocellulose membrane. Meantime, the different results in test zone were recorded when AFB1 positive (100 ng/mL of AFB1) and negative (0 ng/mL of AFB1) samples were applied to ICG strips test treated with the colloidal gold-MAb conjugate. This indicates that the colloidal gold-MAb
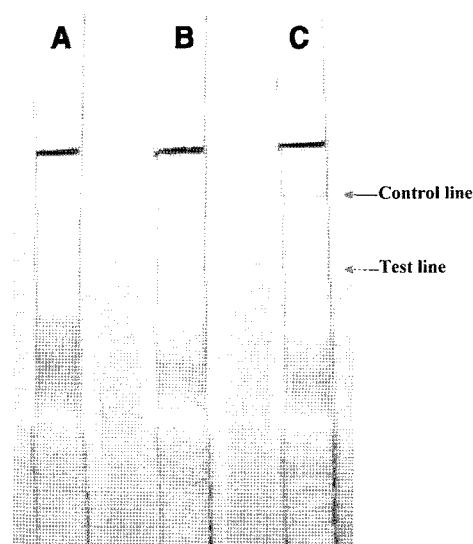

Fig. 3. Evaluation for the suitability of colloidal gold-MAb conjugate in development of ICG strip-test. To determine the non-specific binding of the colloidal gold to immunoreagents on the test and control zones. A, The conjugate pad was treated with colloidal gold-BSA conjugate and performed with PBS containing 10% methanol; B, and C, the conjugate pads were treated with colloidal gold-MAb conjugate; B, result for AFB1 negative test; and C, result for AFB1 positive test.

conjugate could be useful to develop ICG strip-test. In a previous paper, fluorescence spectrometry was used to confirm the colloidal gold-MAb conjugate (30), but this method requires expensive equipments and complicated steps. Therefore, in this study, we suggest simpler method for confirmation of colloidal gold-MAb conjugate.

The sensitivity of a membrane-based assay including ICG strip-test could be controlled by the amount of immobilized immunoreagent (antibody or analyte-protein conjugate) on the membrane and marker (31). If the amount of immobilized immunoreagents and marker are decreased, the sensitivity of the assay will be increased. Therefore, it is key point to find optimal amount of immobilized immunoreagent and marker in the development of membrane-based assay. To develop a sensitive ICG strip-test, the optimal concentrations of immunoreagents were established by titer of AFB1-BSA conjugate against colloidal gold-MAb conjugate of different concentrations. Since the main objective of the ICG strip-test was the qualitative detection of AFB1 without special instruments, it was important that the color intensity of the test zone was strong enough to be seen and enable a clear distinction between negative and positive test. The optimal conditions of ICG strip-test for AFB1 were as follows: 1.5 μg of AFB1-BSA conjugate and 3 μg of anti-mouse IgG were treated in test and control zone on the membrane, and 5 μL of colloidal gold-MAb probe (absorbance at 540 nm was 1.5) was sprayed on conjugate pad. The detection limit of the assay was 0.5 ng/mL of AFB1 (Fig. 4). The results were evaluated visually by eye within 15 min after starting the reaction. ICG assay using polyclonal antibody-gold probe for the detection of AFB1 has been reported (19) but the sensitivity of this (detection limit: 2.5 ng/mL) was lower than the ICG strip-test developed in this study. Cross-reactivity of the ICG strip-
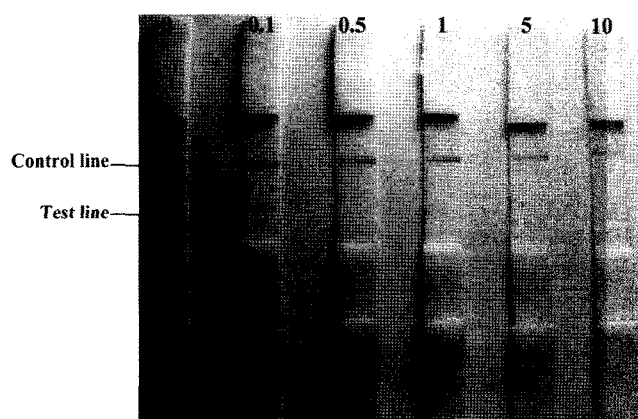
Fig. 4. Detection limit of ICG strip-test for the detection of AFB1. The tests were run 4 times at room temperature using 10% MeOH/PBS spiked with various AFB1 standards. The labels (0, 0.1, 0.5, 1, 5, and 10) indicate the concentration of AFB1 (ng/mL).
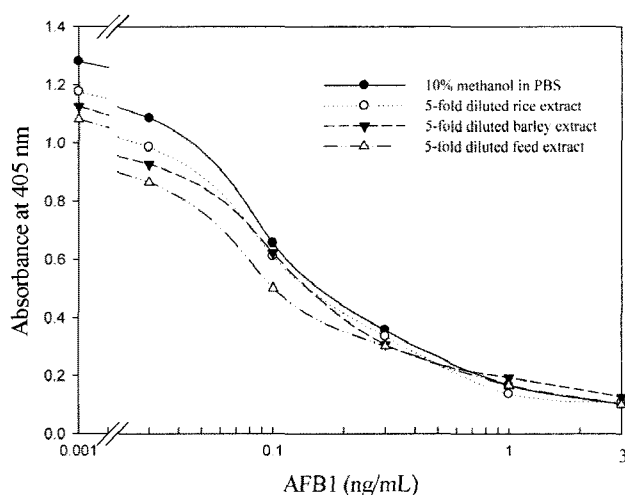

Fig. 5. Assessment of matrix effect from real samples by DC-ELISA.

Table 2. Recovery of AFB1 from spiked samples by DC-ELISA

Sample	Spiked AFB1 concentration (ng/mL)	DC-ELISA[1] Detected AFB1 concentration (ng/mL)	DC-ELISA[1] Recovery (%)
Rice (n=3)	0	ND	NC
	5	5.4±0.4	108±8
	10	8.7±0.6	87±6
	20	17.1±0.8	86±4
	50	50.1±6.4	100±13
	100	94.3±5.2	94±5
Barley (n=3)	0	ND	NC
	5	4.2±0.9	84±18
	10	7.3±0.4	73±4
	20	18.2±1.5	91±7.5
	50	48.5±5.2	97±10
	100	108±14	108±14
Feed (n=3)	0	ND	NC
	5	4.1±0.3	82±6
	10	8.1±0.7	81±7
	20	19.1±1.6	96±8
	50	49±4.6	98±9
	100	92.4±7.2	92±7

[1]ND, not detected; NC, recovery was not calculated.

test was presented in Table 1. The ICG strip-test showed the cross-reactivity for aflatoxins, such as AFB2 (3 ng/mL), AFG1 (1 ng/mL), and AFG2 (3 ng/mL), but no cross-reaction to other mycotoxins was observed.

Assessment of the matrix effect from grain and feed samples Methanol has been generally used to extract AFB1 from food and agricultural products, but this organic solvent will often elute protein and other component which interrupt the interaction between the antibody and free analyte. Because one of the major advantages of immunoassay techniques is their simplicity, the dilution method was used for sample preparation in this study.

To assess the matrix effect from real samples in DC-ELISA, AFB1-free samples of rice, barley, and feed were extracted as described above and diluted from 2- to 10-fold with PBS. AFB1 standard solutions (0-10 ng/mL) were prepared with the diluted extracts and analyzed by DC-ELISA. Although the strong matrix effects were observed in application of original and 2-fold diluted extracts, a similar standard curve with it of buffer (PBS containing 10% methanol) was obtained when the diluted extracts

(from 5- to 10-fold) were applied to DC-ELISA. Thus, we selected 5-fold dilution for sample preparation because increasing dilution may cause decreasing the concentration of AFB1 in real samples. Figure 5 shows the standard curves of DC-ELISA using the buffer and 5-fold diluted extracts. The standard curves using the 5-fold diluted extracts showed the similar standard curve with it of buffer (PBS containing 10% methanol) but these were not identical. Thus, we noticed that a special working buffer is necessary for the practical AFB1 determination in real samples and the 5-fold diluted extracts could be used as working buffer in DC-ELISA. As shown in Table 2, the results showed a good agreement with the amount spiked. The recoveries averaged between 73 and 108% that demonstrate the applicability of DC-ELISA in practical AFB1 determination.

To validate matrix effect from real samples in ICG strip-test, AFB1 standard solutions (0-1 ng/mL) were prepared with extracts diluted from original to 6-fold with PBS and analyzed by ICG strip-test. No red line in all test zones was observed when original extracts of grain (rice and barley) and original and 2-fold diluted extracts of feed were applied to ICG strip-test. Meanwhile, the clear red lines on test and control zone were obtained with the diluted grain (from 2- to 6-fold) and feed (from 4- to 6-fold) extracts. In this study, since increasing dilution cause decreasing the concentration of AFB1 in samples, we selected 2- and 4-fold dilution for grain and feed, respectively. As shown in Fig. 6, AFB1 standard solutions (0, 0.1, 0.5, and 1 ng/mL) prepared with the diluted rice, barley, and feed extracts exhibited same detection limit as that of buffer containing 30% methanol. In the analysis of the spiked samples by
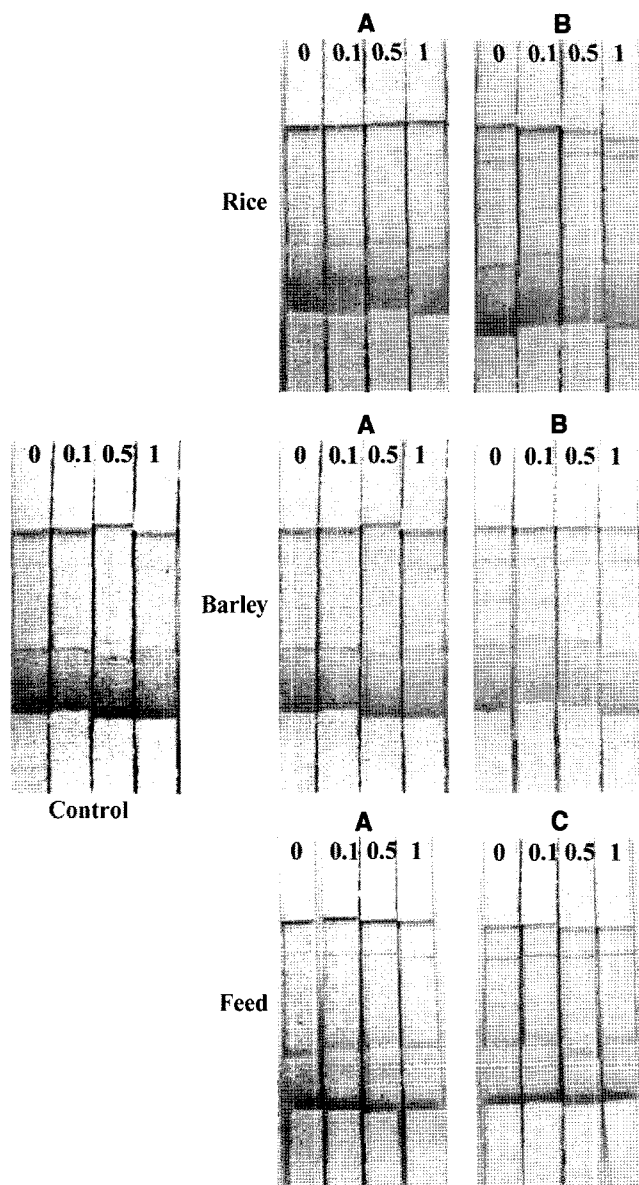
Fig. 6. Assessment of matrix effect from real samples by ICG strip-test. Control indicates that ICG strip-tests were performed with 10% methanol in PBS. A, B, and C were tested with original, 2-, and 4-fold diluted extracts containing AFB1 at different concentration.
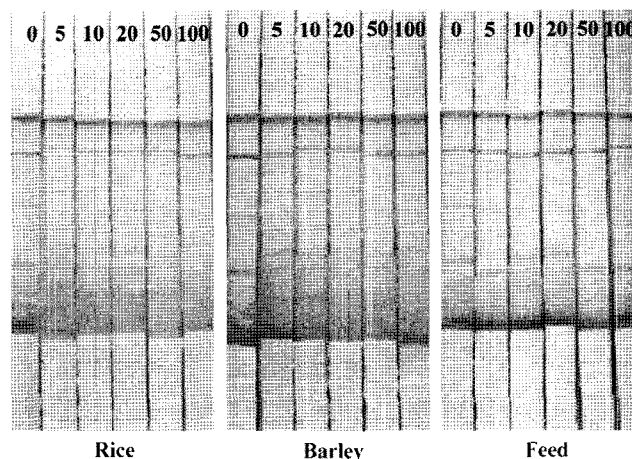

Fig. 7. Analyses of spiked rice, barley, and feed samples with AFB1 at different concentrations by ICG strip-test. The labels (0, 5, 10, 20, 50, and 100) indicate the concentration of spiked AFB1 levels (ng/g).

ICG strip-test, the red lines on test zone appeared at spiked grain with 0 and 5 μg/kg of AFB1 and spiked feed with 0-10 μg/kg of AFB1. However, the red lines on test zone disappeared clearly in application of spiked grain and feed with 10 and 20 μg/kg of AFB1, respectively. After sample preparation, the concentration of AFB1 in the spiked grain (10 μg/kg of AFB1) and feed (20 μg/kg of AFB1) will be all 1 ng/mL. Fortunately, these levels could be detected by ICG strip-test. There, we are certain that ICG strip-test developed in our study possesses sufficient sensitivity for the rapid detection of AFB1 without special instrument within maximum permits established by Korean government (4,5).

Analytical comparison of DC-ELISA and ICG strip-test Both immunoassays developed in this study were

compared with analytical parameter. Even if DC-ELISA (detection limit: 0.004 ng/mL) showed better sensitive than ICG strip-test (detection limit: 0.5 ng/mL), it requires several equipments and complicated steps to complete the measurement of AFB1. The performance of 96 well plate by DC-ELISA could analyze 40 samples within 110 min (coating step: 60 min, competition step: 20 min, color developing step: 20 min, and washing step etc: 10 min) and several 96 well plates could be continuously conducted. Thus, this method could analyze a few hundred samples within its assay time. After sample preparation for DC-ELISA, the AFB1 level in real samples should be diluted 25-fold, so this method could detect 0.1 μg/kg AFB1 level in practical analysis [0.004 μg/kg (detection limit in buffer) ×25 (total dilution time) = 0.1 μg/kg]. Meanwhile, although ICG strip-test is less sensitive than DC-ELISA, this method is more rapid (assay time: 15 min), non-instrumental, easy to complete the measurement of AFB1. This assay requires one step (sample application) to complete AFB1 determination and possesses higher sample throughput than DC-ELISA because this method could be simultaneously performed (Table 3). After sample preparation for ICG strip-test, AFB1 levels in grain and feed samples should be diluted, but the Korean guideline could be detected sufficiently by ICG strip-test.

Monitoring of AFB1 in natural samples A total of 172 samples (64 rice, 43 barley, and 45 feed samples) were analyzed by both ICG strip-test and DC-ELISA, and these results compared with those obtained by HPLC were shown in Table 4. Of the 172 samples, 18 samples (1 of rice, 8 of barley, and 9 of feed) and 5 feed samples were found to be positive of AFB1 by DC-ELISA and ICG strip-test, respectively. Meanwhile, 8 (2 of barley and 6 of feed) of 172 samples were found to be AFB1 contamination by HPLC. The AFB1 positive samples by ICG strip-test and HPCL belonged to the list of AFB1 positive samples by DC-ELISA. On the other hand, the analytical results of ICG strip-test were in a good agreement with those obtained by HPLC. Especially, the results of ICG strip-test and HPLC at 1 μg/kg of AFB1 were identical. Strangely,

Table 3. Analytical parameters of DC-ELISA and ICG strip-test

Analytical parameter	DC-ELISA	ICG strip-test
Competitor	AFB1-HRP conjugate	Colloidal gold-MAb conjugate
Detection limit (ng/mL) in buffer	0.004	0.5
Dynamic range (ng/mL)	0.005-5	-
Assay steps	6	1
Assay time	110 min (about 2 hr)	15 min
Sample throughput in assay time	A few hundred samples	A few hundred samples, but more than DC-ELISA
Dilution time for sample extracts	5-fold for rice, barley, and feed	2-fold for rice and barley, 4-fold for feed
Practical detection limit in real sample	0.1 µg/kg in rice, barley, and feed	10 µg/kg in rice and barley, 20 µg/kg in feed

Table 4. Results of AFB1 analysis in rice, barley, and feed samples by DC-ELISA, ICG strip-test, and HPLC

Samples (tested)	List of positive samples	Results for AFB1[1]		
		DC-ELISA	ICG strip-test	HPLC (µg/kg)
Rice (64)	GGR6	+	-	-
Barley (43)	GGB2	+	-	-
	GWB5	+	-	-
	CBB5	+	-	-
	CNB4	+	-	0.37
	GBB1	+	-	
	GBB7	+	-	0.78
	JBB5	+	-	-
	JNB5	+	-	-
Feed (65)	Ch-ox1	+	+	1.33
	Ch-ox4	+	-	-
	Ch-ox5	+	-	-
	Ch-ox7	+	+	3.76
	Jn-ox2	+	+	15.58
	Jn-ox4	+	+	3.21
	Jn-fi4	+	-	0.48
	Gi-ox1	+	+	1.45
	Gi-pi2	+	-	-

[1]+, Positive, - negative results in the assay.

ICG strip-test could detect <10 µg/kg of AFB1 in real samples. The reason is that the contamination with other aflatoxins was frequent if the samples were contaminated with AFB1 and maybe ICG strip-test detected other aflatoxins. One (Jn2) feed sample showed the highest AFB1 level (15.58 µg/kg) but AFB1 levels in all real samples were lower than Korean guide line. According to results, 2 immunoassays developed have practical advantages, especially food and agricultural monitoring with high sample throughput. Besides, these methods possess a potential as a rapid and cost-effective screening tool for AFB1 or aflatoxins determination in real samples.

In conclusion, the importance and application of ELISA in AFB1 monitoring have grown significantly. In recent years, the ICG strip-test has been investigated and used for mycotoxins monitoring. In this study, 2 immunoassays, such as DC-ELISA and ICG strip-test were developed and compared for the rapid detection of AFB1 in real samples.

Although, DC-ELISA possesses higher sensitivity than ICG strip-test, this requires long assay time and complicated steps for AFB1 determination. Meantime, the sensitivity of ICG strip-test was lower than DC-ELISA, but it is sufficient for the detection of maximum permit limit established by Korean government without any instruments and easy to perform. Besides, the ICG strip-test requires only 15 min to analyze sample. Good recoveries were obtained from the most spiked samples by DC-ELISA and the spiked grain and feed samples with 10 and 20 µg/kg of AFB1 were confirmed to be AFB1 positive by ICG strip-test. Comparative analyses of naturally contaminated rice, barley, and feed samples performed by DC-ELISA, ICG strip-test, and HPLC showed a good agreement (Table 4). Both immunoassays were highly specific and reproducible and could be applied to analysis of AFB1 in real samples. Finally, DC-ELISA and ICG strip-test possess practical advantages for AFB1 or aflatoxins screening because they have cross-reactivity to aflatoxins. With respect to its overall speed and simplicity, ICG strip-test is better than DC-ELISA and instrumental analysis. However, if a higher sensitivity and lower detection limits are needed, DC-ELISA is better suitable.

Acknowledgments

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