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Development of an Immunoassay-Based Lateral Flow Dipstick for the Rapid Detection of Aflatoxin B_1 in Pig Feed

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The aim of this work was to develop an immunoassay-based lateral flow dipstick for the rapid detection of aflatoxin B_1 in pig feed. The test consisted of three main components: conjugate pad, membrane, and absorbent pad. The membrane was coated with two capture reagents, that is, aflatoxin B_1−bovine serum albumin conjugate and rabbit anti-mouse antibodies. The detector reagent consisted of colloidal gold particles coated with affinity-purified monoclonal anti-aflatoxin B_1 antibodies, which saturated the conjugate pad. A comparison of several extraction methods for the pig feed matrix is presented. A mixture of methanol/water (80:20, v/v) gave the best recoveries. After sample extraction and dilution, the dipstick was put in the sample solution at the conjugate pad side and developed for 10 min. Analyte present in the sample competed with the aflatoxin B_1 immobilized on the membrane for binding to the limited amount of antibodies in the detector reagent. Thus, the line color intensity of an aflatoxin B_1-positive dipstick is visually distinguishable from that of an aflatoxin B_1-negative sample. The visual detection limit for aflatoxin B_1 is 5 μg/kg. The major advantages of this one-step striptest are that results can be obtained within 10 min and that all reagents are immobilized on the lateral flow dipstick.

KEYWORDS: Aflatoxin B_1; lateral flow; dipstick; rapid detection assay; pig feed

INTRODUCTION

Aflatoxins are a group of toxic secondary metabolites mainly produced by the fungi *Aspergillus flavus*, *Aspergillus nomius*, and *Aspergillus parasiticus* growing in a wide range of foods and feedstuffs. Although ∼20 aflatoxins have been identified, only 4 of them, that is, the aflatoxins B_1, B_2, G_1, and G_2, occur naturally and are significant contaminants of a wide variety of feeds. Aflatoxin B_1 or 2,3,6a,9a-tetrahydro-4-methoxycyclopenta[*c*]furo[3′,2′:4,5]furo[2,3-*h*][1]benzopyran-1,11-dione (*1*) is usually found in the greatest concentration in feed and was classified as a carcinogenic substance of group 1 by the International Agency for Research on Cancer (IARC) (*2*, *3*).

Pre- or postharvest contamination of various feeds by mycotoxigenic fungi is a common problem for both public health and economy. Approximately 25% of the world's food supply is contaminated by mycotoxins annually. Although the actual resultant economic losses are difficult to determine accurately, they are likely to be high (*4*).

Aflatoxins represent a main threat worldwide. It is important to restrict the intake of aflatoxin B_1 by humans and animals, and therefore regulations have been set. However, the legal limits vary significantly from country to country (e.g., for aflatoxins from 0 to 50 ng/g). Regulatory limits have been set and drafted in the European Union to harmonize the internal market. In Commission Directive 2003/100/EC maximum aflatoxin B_1 contents are fixed for all kinds of animal feeds. For example, for complete feedstuffs for dairy animals and other complementary feedstuffs the level has been set at 5 μg/kg, which is the most stringent limit (*5*). These are relatively low levels compared to the global situation (*6*).

The early detection of mycotoxin-contaminated lots is essential. Screening tests should be carried out for control at all stages of food and feed production, so that contaminated ingredients can be rejected or identified for feeding animals or humans (*7*−*9*). To obtain correct analytical results, representative sampling is of utmost importance. This is a challenge for mycotoxins, because they are distributed heterogeneously throughout the sample in so-called "hot-spots" (*10*). Methods of analysis and sampling for the official control of aflatoxins are described in European Directive EC/53/1998 (*11*).

Aflatoxin analysis can be conducted using validated analytical methods with good sensitivity and precision such as thin-layer

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chromatography (TLC), high-performance liquid chromatography (HPLC), and enzyme-linked immunosorbent assay (ELISA). Most of these methods are time-consuming or use expensive equipment, require well-trained personnel, and can only be employed in laboratories (*12*, *13*). Therefore, there is a need for rapid and cheap but reliable methods that can be conducted and interpreted by users who are close to the site of contamination. More and more on-site immunological techniques such as dipstick, immunochromatography, and immunofiltration are gaining interest in the area of mycotoxin detection in feed and food. Most of them are basically designed as visual tests (*12*, *13*). Membrane substrates are valuable solid phases in immunoassay for application in the field (*14*). With this technique it is possible to use simple and minimal manipulations and to provide accurate results with little or no instrumentation.

This study was designed to develop a portable lateral flow dipstick for the rapid on-site detection of aflatoxin B_1 in pig feed. Therefore, an efficient extraction method has been developed with good recoveries and a visual read-out lateral flow dipstick technique. This method is intended as a screening facility, complementary to the conventional quantitative method, but not replacing it. This approach results in saving both time and costs, making it useful for companies without access to sophisticated equipment to meet the requirements of the rapidly evolving EU legislation.

MATERIALS AND METHODS

Reagents. Aflatoxin B−bovine serum albumin conjugate (BSAC) and Tween 20 were purchased from Sigma Aldrich Co. (Bornem, Belgium). The rabbit anti-mouse immunoglobulins (RAM) (Ig) (protein concentration = 2.5 g/L) were from Dakocytomation (Glostrup, Denmark). Sucrose was of ACS reagent grade (ICN Biochemicals, Cleveland, OH), and methanol and acetonitrile were of high-performance liquid chromatography grade (VWR International, Poole, U.K.). Monoclonal antibodies from mice against aflatoxin B_1 were produced and characterized by the Institute for Animal Sciences, Agricultural Biotechnology Center, Gödöllö, Hungary. The antibody was an IgG2 (protein content = 1 g/L) with λ light chain. It cross-reacted with aflatoxin B_2 (76%), aflatoxin M_1 (79%), aflatoxin M_2 (33%), aflatoxin G_1 (55%), and aflatoxin G_2 (6%), but not with aflatoxins B_{2a} and G_{2a}. The antibody was conjugated to colloidal gold particles (40 nm) by British Biocell (Cardiff, U.K.), resulting in colloidal gold-labeled antibodies (CGC) with an optical density (OD) of 10.2. Phosphate-buffered saline (PBS) (0.01 M, pH 7.4) was used to make the BSAC buffer (PBS−0.07% methanol), the CGC buffer (PBS−25% sucrose−0.5% Tween 20), and the diluted sample extract. Water was obtained from a Milli-Q gradient system (Millipore, Brussels, Belgium). Stock solutions of aflatoxin B_1 (1 mg/mL) and aflatoxin B_1 standard solutions (10 and 1 ng/μL) were prepared in methanol and stored at −20 °C. Pig feed samples (complete sow-gestation feed and complete sow-lacto feed) were obtained from Vanden Avenne (Ooigem, Belgium). Compound feed certified reference materials (9.3 μg/kg aflatoxin $B_1 \pm 0.5$ and <1 μg/kg aflatoxin B_1) came from the Institute for Reference Materials and Measurements (IRMM), European Commission, Geel, Belgium.

Various materials were tested for their suitability as conjugate pad: Accuwick Ultra (Pall, Saint Germain en Laye, France), glass fiber grade 8975 (Pall), and 33 glass fiber conjugate pad (Schleicher & Schuell, Dassel, Germany). The absorbent pads (cellulose absorbent papers) and the membrane [laminated (adhesive polyester) Predator membrane] were also obtained from Pall.

Apparatus. For the extraction a mini shaker S03 was used from Orbital (Stuart Scientific, Surrey, U.K.). A Centra-MP 4 centrifuge was obtained from International Equipment Co. (Needham, MA). A Linomat 5 spotting machine from Camag (Mettler-Toledo, Zaventem, Belgium) was used. For the analysis of extraction recoveries an HPLC system was used. Samples were injected on the 250 × 4.6 mm i.d., 5 μm reversed-phase Discovery C_{18} analytical column (Supelco, Bellefonte, PA) by means of a Waters 717 plus autosampler (Waters, Milford, MA). Mobile phase was pumped through the column with a Waters 600 pump equipped with Millenium software for data processing. Detection was done by a Waters 474 scanning fluorescence detector at λ_{ex} 274 nm and λ_{em} 440 nm.

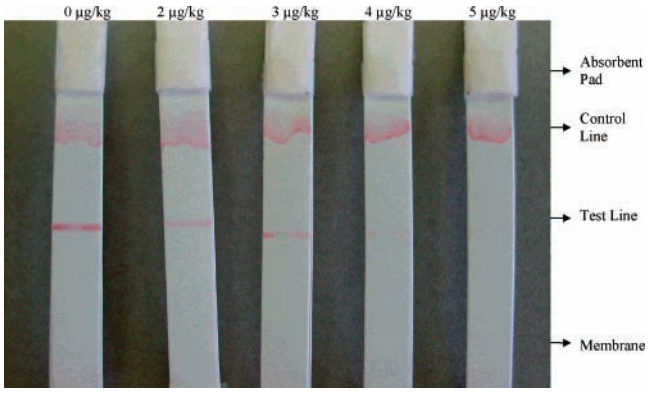

Figure 1. Development of lateral flow dipsticks for aflatoxin B_1 in pig feed samples at different concentrations. The control line was manually spotted and the test line with Linomat 5. The visual detection limit is on 5 μg/kg.

Procedure. *Preparation of Lateral Flow Dipsticks.* First the membrane strips (5 × 50 mm), consisting of a backing plate (adhesive polyester) on which the membrane was pasted, were manually spotted with the control capture reagent (undiluted RAM) and test capture reagent (BSAC buffer: 0.052 mg/mL BSAC in PBS and 7% MeOH) at a volume of 1 μL/0.5 cm membrane width (**Figure 1**). The strips were dried for 1 h at room temperature. When using the Linomat 5, capture lines were separately sprayed at two different places near one end (top) of the 210 × 50 mm membrane sheet with a volume of 1 μL/0.5 cm membrane width, leaving a 5 mm space between the two lines (**Figure 1**). After drying, the membrane sheet was divided into 5 mm × 50 mm strips using scissors. A conjugate pad with a size of 5 × 30 mm was saturated with 15 μL of OD1 CGC buffer prepared by dilution of colloidal gold-labeled antibodies OD 10.2 with PBS containing 25% (w/v) sucrose and 0.5% (w/v) Tween 20. The conjugate pad was dried for 1 h at room temperature. An absorbent pad was cut in sections of 0.5 × 2 cm and fixed with tape at the far end of the lateral flow dipstick in the direction of the flow. Afterward, the impregnated conjugate pad was placed at the opposite end of the lateral flow dipstick (i.e., at the origin of the sample flow). The lateral flow dipstick was ready for use in assay and stored under dry conditions (desiccator) at room temperature until use.

Lateral Flow Dipstick Immunoassay. (a) Sample Preparation. On the basis of the legislation for aflatoxin B_1 contamination in feed, it was our objective to set the visual detection limit for aflatoxin B_1 at 5 μg/kg of feed (*5*). Five-gram blank pig feed portions, analyzed with the HPLC method, were fortified with 0, 4, 5, or 6 μg/kg aflatoxin B_1 standard solution (1 ng/μL in methanol) 1 day prior to extraction. After 1 day, the sample was homogenized by manual shaking.

After the samples had been mixed with 15 mL of extraction solvent ($MeOH/H_2O$, 80:20), they were manually shaken for 5 min. After dilution of 2 mL of the raw extract with 2.8 mL of PBS, the final methanol concentration was 33%. This diluted extract was directly used in the assay without further cleanup.

(b) Immunoprocedure. Diluted extract (250 μL) was pipetted in the wells of an ELISA microtiterplate. The dipstick was dipped in the extract solution at the conjugate pad side. The extract was allowed to flow over the conjugate pad. As a result, the CGC immobilized on the conjugate pad dissolved again in the extract. In a sample containing aflatoxin B_1, the toxin was able to bind with these CGC antibodies and formed an analyte−detector complex. In the case of a blank sample the CGC remained free. The solution began to move up the membrane by means of the absorbent pad. Passing the test line, free CGC antibodies bound to the immobilized aflatoxin B_1 on the membrane and formed a pink line. The analyte−detector complex passed the test line because the complex had no binding sites available. The smallest

toxin concentration that resulted in no color intensity was considered to be the visual detection limit. The control capture line was a check for the good performance of the test to ensure that the CGC antibodies migrated throughout the system. If no control line was present, the test was considered to be invalid. Thus, the appearance of two pink lines (test and control capture line) meant that the sample should be declared compliant (i.e., aflatoxin B_1 concentration < 5 μg/kg). When only a single line (control capture line) appeared, the sample should be declared noncompliant (i.e., aflatoxin B_1 contamination ≥ 5 μg/kg).

HPLC Method Used for Extraction Solvent Comparison. To compare the extraction solvents, recoveries were evaluated. Therefore, the amount of toxin found after analysis in the sample fortified before extraction was divided by the amount of toxin found after analysis in the sample fortified after extraction and cleanup.

(a) Sample Preparation. The sample preparation was performed using a modification of the method of Vicam (*15*). Twenty-gram portions of pig feed were prepared for the comparison of different extraction solvents. Half of the samples were fortified with aflatoxin B_1 (8 μg/kg) 1 day prior to analysis. The other half of the samples were blank. The extract of these blank samples was fortified at the 8 μg/kg aflatoxin B_1 level after cleanup (AflaTest immunoaffinity columns, Vicam, Watertown, MA).

All samples were extracted with 50 mL of extraction solvent by shaking them on the mini shaker for 30 min. After centrifugation (10 min, 3600 rpm), 10 mL of the clear supernatant was diluted with 40 mL of PBS so that a final organic phase concentration of $\sim 16 \pm 2\%$ was obtained. The cleanup was performed with AflaTest immunoaffinity columns. The 50 mL diluted extract was passed over the column at a rate of 1−2 drops/s. After the column had been washed with 5 mL of water, the toxin was eluted with 4 mL of methanol by gravity.

(b) Analysis. The mobile phase consisted of MeCN/H_2O (55:45, v/v) and had a flow rate of 1.0 mL/min. The fluorescence detector excitation and emission wavelengths were set at 360 and 440 nm, respectively. Fifty microliters of purified sample extract was injected and analyzed. Afterward, peak areas were integrated to calculate the amount of toxin present in the sample.

RESULTS AND DISCUSSION

Comparison of Different Extraction Solvents. The aim of this study was to develop a rapid field test. Therefore, an extraction method that emphasized speed and minimal manipulations was desired in this field test approach. If more toxin is extracted from a sample, resulting in a higher toxin concentration in the extract, a lower visual detection limit can be reached. Therefore, different extraction methods were compared to find out which one gave the best recoveries and was the easiest in handling.

Methanol or acetonitrile in combination with water is often used for the extraction of aflatoxin B_1 from cereals (*2*, *16*, *17*). Acetonitrile was not used as the extraction solvent in this approach because of its toxicity (*18*). Sometimes NaCl is added to the samples before extraction of other mycotoxins from cereals (*12*, *19*). Therefore, an extraction method with MeOH/H_2O and addition of NaCl was also carried out and compared for aflatoxin B_1 extraction efficiency.

Fortified samples extracted with several extraction solvent compositions showed different recoveries. MeOH/H_2O (80:20, v/v) gave the best recovery, that is, $86.4 \pm 12.8\%$ ($n = 4$). Recoveries of $80 \pm 5.3\%$ ($n = 4$) and $66 \pm 1.7\%$ ($n = 4$) were found for 90:10 and 70:30 MeOH/H_2O, respectively. The recoveries of the extraction method with the addition of NaCl were not as good as in the other method and required one step more [extractions of 90:10, 80:20, and 70:30 MeOH/H_2O gave recoveries of $75 \pm 7.6\%$ ($n = 4$), $80 \pm 5.2\%$ ($n = 4$), and $73 \pm 6.9\%$ ($n = 4$), respectively]. Therefore, MeOH/H_2O (80:20, v/v) extraction was preferred to develop the lateral flow dipstick. As no background due to matrix interferences appeared on the membrane of the lateral flow dipstick, no further cleanup was necessary.

Development of the Lateral Flow Dipstick Test. *Components of the Test.* The lateral flow dipstick is constructed with three main elements: a conjugate pad, a membrane, and an absorbent pad. The Predator laminated membrane is often used for the development of lateral flow tests, and therefore this type was also applied as membrane in the lateral flow dipstick test. Because there were no problems of background and there was no flow obstruction, it was not necessary to try other membrane types. The conjugate pad acts as a reagent delivery vehicle for the assay CGC. From the tested materials the Pall glass fiber conjugate pad gave the best release and appeared to be the most appropriate material for this assay. Absorbent pads have the ability to control sample flow over the membrane by pulling immunoreagents over the test and control capture lines and by trapping the excess immunoreagents. This material should be absorbent and have a high volume capacity (5 mm $\times$ 30 mm). A cellulose pad was chosen. It was possible to use the test without sample pad and to insert the conjugate pad directly in the sample, like a dipstick test.

Optimization of Working Dilutions. The purpose of a lateral flow dipstick is to allow visual evaluation of the presence or absence of the analyte. The sensitivity of the membrane-based assay can be controlled by adjusting the amount of immobilized BSAC on the membrane and the OD of the CGC. With lower amounts of aflatoxin B_1−BSA conjugate, sensitivity will increase but the color intensity will decrease. The visual detection limit (VDL) of the aflatoxin B_1 lateral flow dipstick is defined here as the amount of aflatoxin B_1 in the sample solution at which no test capture line is visible. As the EU maximum limits for aflatoxin B_1 in pig feed are established at 5 μg/kg, the VDL of the strip test was also fixed at 5 μg/kg (*5*).

The optimal working dilutions were determinated by trial and error. A dilution of the CGC OD 1 resulted in intense pink capture lines for a blank sample without giving a pink background on the membrane. The ultimate composition of the detector reagent mixture on the conjugate pad was as follows: 13.5 μL of PBS [25% sucrose (m/v) and 0.5% Tween 20 (v/v)] and 1.5 μL of OD 10.2. By adding sucrose, the viscosity of the detector mixture increased, resulting in a slower moving flow front. This made sure that there was enough reaction time between the detector mixture and the capture lines. There was also a good solubilization of the detector mixture from the conjugate pad and a better protection of the CGC in the mixture. This resulted in intensely pink capture lines. These were stained uniformly by the addition of 0.5% Tween 20 as surfactant. The RAM for the control capture line was used undiluted. The test capture line (BSAC) was diluted to a final concentration of 0.052 mg/mL in PBS. By adding 7% methanol (v/v) in this mixture, the BSAC became less soluble, without causing denaturation or precipitation, and could adsorb better onto the membrane (*20*). The test capture line of a blank sample had the same intensity as the control capture line.

The amounts of test detector reagent and test capture reagents were set so that the test capture line was no longer visible from 5 μg/kg aflatoxin B_1 on. In a sample fortified with 5 μg/kg aflatoxin B_1 or more, all CGC antibodies were occupied by the aflatoxin B_1 of the sample; hence, no free CGC antibodies remained. Therefore, no pink line appeared on the test line. The complex was thus only trapped by the control line, and a pink band developed. Also, CGC of samples containing <5 μg/kg

Table 1. Results of the Intralaboratory Validation[a]

		quantitative		
		⩾5 ppb	<5 ppb	total
qualitative	positive (= noncompliant)	47 (tp)	4 (fp)	**51**
	negative (= compliant)	0 (fn)	37 (tn)	**37**
	total	**47**	**41**	**88**

[a] ⩾5 ppb, concentrations 5 ($n = 12$), 6 ($n = 12$), 7 ($n = 12$), or 10 ($n = 11$) μg/kg; <5 ppb, concentrations 0 ($n = 13$), 3 ($n = 14$), or 4 ($n = 14$) μg/kg; tp, true positive; fp, false positive; fn, false negative; tn, true negative.

aflatoxin B_1 bound to RAM and formed a pink control line. Excess CGC was trapped by the absorbent pad. After 10 min, the test result was evaluated. The test capture line of a blank sample showed the most intense pink color because of the inverse relationship between toxin concentration and color development. However, 3 μg/kg aflatoxin B_1 already resulted in a considerable decrease of the test capture line intensity as compared with the test capture line obtained with samples containing no aflatoxin B_1 (**Figure 1**).

When a lateral flow dipstick test on which capture lines were spotted manually was developed, lines did not stain uniformly. By use of the Linomat 5, lines did develop uniformly and the result could be read-out more easily. The best result was obtained after a run of 10 min, making this test a rapid field test.

Validation of the Lateral Flow Dipstick. There are no general validation guidelines available for qualitative analytical methods (*21*).

This assay is a qualitative method in which the eye is used to record and interpret the response. The test provides a yes/no response indicating that aflatoxin B_1 is present or not above the VDL (*21*). The VDL in this qualitative method is the lowest aflatoxin B_1 concentration level that inhibits color development, with a certain probability of error (usually 5%) (*21*, *22*). An intense pink line appeared for blank pig feed samples. The color intensity decreased with increasing aflatoxin B_1 concentrations, and no pink line appeared at aflatoxin B_1 concentrations of 5 μg/kg or more.

An intralaboratory validation was performed determining the following performance characteristics: specificity, sensitivity, false noncompliant (false positive), and false compliant (false negative) rates. These parameters were determined at several concentrations (0, 2, 3, 4, 5, 6, 7, and 10 μg/kg) including the level of visual detection limit on different days. The sensitivity in qualitative methods is the ability of a method to detect truly positive (tp) samples as positive and is calculated as the number of truly positive tests divided by the sum of the number of known positives [= true positive results + false negative (fn) results] $[(t_p/t_p + f_n) \times 100]$ (*21*). The lateral flow dipstick had a sensitivity of 100% ($n = 88$) (**Table 1**). The specificity is the ability of a method to detect negative samples as negative and is calculated as the number of truly negative (tn) tests divided by the number of known negatives [= true negative results + false positive (fp) results] $[(t_n/t_n + f_p) \times 100]$ (*21*). The lateral flow dipstick had a specificity of 90% ($n = 88$) (**Table 1**). The false compliant (false negative) rate was calculated as the number of false compliants divided by the sum of the false compliants and true noncompliants ($f_n/f_n + t_p$). The false noncompliant (false positive) rate was calculated as the number of false noncompliants divided by the sum of false noncompliants and true compliants ($f_p/t_n + f_p$). The assay was accurate and reliable, giving no false compliant results and only a low percentage (10%) of false noncompliant results. Samples scoring noncompliant during the screening always will require chromatographic confirmation.

The lateral flow dipstick was also evaluated with certified reference materials. All tests with compound feed containing 9.3 μg/kg $\pm$ 0.5 aflatoxin B_1 ($n = 7$) showed one pink line, whereas blank compound feed ($n = 5$) showed two pink lines.

The lateral flow dipstick is easy to perform. Results can be obtained within 10 min without the need of expensive handling or equipment. The integrated control serves as a confidence on the functionality of the assay as well as acting as the standard color intensity reference for test samples. The test has been tuned for a VDL of 5 μg/kg for aflatoxin B_1 in pig feed, providing a yes/no response allowing the discrimination of "noncompliant" samples from the samples. It can be concluded that the described assay format offers potential as a reliable on-site screening tool.

ABBREVIATIONS USED

BSA−aflatoxin B_1, bovine serum albumin−aflatoxin B_1; BSAC, bovine serum albumin conjugate; CGC, colloidal gold conjugate; VDL, visual detection limit; OD, optical density; PBS, phosphate-buffered saline; RAM, rabbit anti-mouse immunoglobulins.

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