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ORIGINAL PAPER

Colloidal gold based immunochromatographic strip for the simple and sensitive determination of aflatoxin B1 and B2 in corn and rice

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Abstract We have developed a simple and fast immunochromatographic test strip for the simultaneous quantitation of aflatoxin B_1 and aflatoxin B_2 in corn and rice. The strip contains three pads (sample, conjugate, and absorbing pad) and uses the respective polyclonal antibodies immobilized on gold nanoparticles. Matrix interferences were minimized by application of fugacity theory. Clean-up of samples and pretreatment of strip pads is not required. The visual detection limit is 0.1 ng mL^{-1}, and the process can be completed within 5 min. Out of 113 natural samples, 16 rice and 27 corn samples (38% in total) were aflatoxin positive and the test results were confirmed by HPLC. The strip shows, however, high cross reactivity to aflatoxins G_1, G_2, and M_1. We consider this strip to possess wide applicability because of its ease of use, sensitivity, stability, and low cost.

Keywords Aflatoxin B_1, B_2 · Corn · Rice · Colloidal Gold Immunochromatographic strip · Fugacity · Kenya

Introduction

Aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), and G_2 (AFG_2) are the best known of the fourteen aflatoxins, produced under particular field conditions of crop growth or storage by some strains of *Aspergillus flavus, A. parasiticus* and *A. nomius;* these fungi contaminate agricultural products. High-level exposure in human food or stock feed may cause acute effects including rapid death or long-term chronic effects such as mutagenicity, allergic reactions, liver cirrhosis, induction of tumors, impaired central nervous system, immunosupression and micronutrient uptake interference among other disorders [1–3]. Aflatoxins have been listed as group I carcinogens by the International Agency for Research on Cancer (IARC) [4]. The acceptable levels of aflatoxin vary from country to country with the European Union having the lowest values at 2 μg kg^{-1} for AFB_1 and 4 μg kg^{-1} for total aflatoxins in groundnuts, nuts, dried fruits, and cereals [5]. Kenya has set AFB_1 levels in grains and all food for human consumption at 10 μg kg^{-1} [6, 7].

Kenya is the only African country documented for recurrent outbreaks of acute aflatoxicosis resulting from contamination in corn (maize) and other cereals, with the largest outbreak reported in 2004, with 317 cases and 125 deaths. The affected corn contained as much as 4,400 μg kg^{-1} aflatoxin B_1, which is 440 times higher than the 10 μg kg^{-1} set limit [6]. Although outbreaks of aflatoxicosis have reduced, chronic exposure in market products is still high. Survellaince of maize from 2005 to 2007 in Eastern Kenya [7] and 2006 to 2009 in Nairobi markets [8] still recorded aflatoxin levels above the set limit. Corn and rice are two important staple foods with a dietary intake of 400g per person per day [2]. Overdependence on corn coupled with a warm tropical climate throughout the year and poor storage conditions often results in aflatoxin food contamination and poisoning culminating in several fatalities in various parts of Kenya, especially during times of food scarcity [9, 10]. Campaigns such as good agricultural practices, good storage, proper drying, and safe livestock feeds have been preferred as the easiest methods for the control of

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mycotoxigenic fungi and resultant toxins [6, 9]. Although campaigns on good agricultural practices have shown reduction in aflatoxicosis fatality occurrences in West Africa and elsewhere [11], contamination of grains has not reduced as shown in a recent study by Okoth and Kola [8].

There are a variety of well established methodologies, often based on their fluorescence, for analyzing aflatoxins and other mycotoxins in different foodstuffs; these include thin layer chromatography, ultra-pressured layer chromatography, immunoaffinity chromatography, high-performance liquid chromatography, near infrared spectroscopy, microbial tests and immunoassay [12–14]. However, these widely accepted methods have serious limitations in resource-poor countries. They are generally laborious, expensive both in the initial and maintenance costs, need extensive sample preparation, are not suitable for on-site and field screening, and cannot be easily performed by unskilled personnel to whom all such methods are inaccessible. There has been increasing demand in developing regions such as South-East Asia, Africa, and the Middle East, who are the major exporters of food and agricultural products, that are very susceptible to aflatoxin contamination, to develop simple and cheaper methods, more pertinent to developing countries [15]. The ICG techniques otherwise referred to as lateral-flow assays, have well elucidated advantages of simplicity, field and on-site usage, no need for technical skill to interpret results and above all, more affordable than other conventional methods [12, 14, 16–18]. However, even with these cumulative benefits this technology is still not widely used. This research paper aims to satisfy this demand by developing a simple immunochromatographic strip (ICG) for AFB_1 and AFB_2.

Experimental

Reagents and chemicals

All chemicals used including; goat anti-rabbit IgG, chloroauric acid ($HAuCl_4$), polyethylene glycol (PEG) 20000, AFB_1, AFB_2, AFM_1, AFG_1, AFG_2 standards, bovine serum albumin (BSA), AFB_1-BSA conjugate, potassium carbonate, sodium azide, sodium chloride, and methanol, were obtained from Sigma Chemical Co. (St. Louis, MO, USA, http://www.sigmaaldrich.com). Protein A-Sepharose 4B was purchased from Amersham Biosciences (Uppsala, Sweden, www.gelifesciences.com). Double distilled water was produced using a Millipore Milli-Q water system (Bedford, MA, USA, www.millipore.com). All other reagents used in this experiment were purchased from Tianjin No.1 Chemical Reagent Factory (Tianjin, China, www.reagent-1.com). Nitrocellulose membrane (capillary flow of 180s/4cm) was purchased from Millipore (Bedford, MA, USA, www.millipore.com). Sample pads, conjugate pads, absorbent pads, polyvinyl chloride sheets and filter paper were purchased from Kinbio Tech Co. (Shanghai, China, www.kinbio.com). Multiskan Spectrum was purchased from Thermo Labsystems (Vantaa, Finland, www.thermo.com).

Polyclonal antibody B_1+B_2, + KLH was developed by one of us, I. Kennedy. All standard solutions for AFB_1, AFB_2, AFG_1, AFG_2 and AFM_1 were prepared by dilution of stock solutions of these compounds (1 mg mL^{-1} in methanol), whereas the coating conjugate was diluted 1 mg mL^{-1} in phosphate buffer (PB). All chemicals and organic solvents used were of analytical reagent grade. Corn and rice samples were obtained from leading stores and open air markets from Nairobi, Kenya and its environs. The grains and flours were purchased and carefully repackaged into 200 g quantities, sealed and transported to the Key Laboratory of Food Nutrition and Safety, Tianjin University of Science and Technology, China. The samples were stored at 4 °C until analyzed.

Synthesis of nano-colloidal gold-probe

We prepared colloidal gold of 20 nm as described by Sheng et al. [19]. Briefly the pH for colloidal gold solution and the optimal amount of antibody for conjugation was determined as a series of small amounts of colloidal gold titrated with different amounts of 0.2 M K_2CO_3 (pH adjustor) or of purified antibody (1 mg mL^{-1}); the tube that retained the best color of the colloidal gold depicted the optimal pH or the amount of antibody needed for stabilization of colloidal gold respectively. 10 mL of colloidal gold was then measured into a test-tube and pH adjusted to 8–9. The solution was then distributed into 1 mL tubes each and 40 μL of purified antibody (1 mg mL^{-1}) added to each milliliter. The tubes were shaken gently for 30 s and incubated under room temperature (15–30 °C) for 1 h, after which 20 μL of BSA (20%) and 10 μL of PEG 20000 (20%) was added to each milliliter for blocking residual surfaces of the nano-colloidal gold particles and for stabilizing the conjugates respectively. The solution was further incubated for 5 min and centrifuged at 2000 r/min at 4 °C for 15 min; 1 mL supernatant was drawn into new tubes and re-centrifuged at 10000 r/min at 4 °C for 30 min. 950 μL supernatant was then drawn from each tube and discarded. The precipitates were mixed together and diluted with 1:10 (v/v) working buffer (0.1 mol L^{-1}Tris-HCl buffer (pH 8.5) containing 5% sucrose, 0.5% BSA and 0.05% NaN_3). The solution was then immediately sprayed on the colloidal gold pad and left to dry in an electric blast drying vacuum oven overnight at 37 °C. The dried pads were stored in a dryer under room temperature until ready for use.

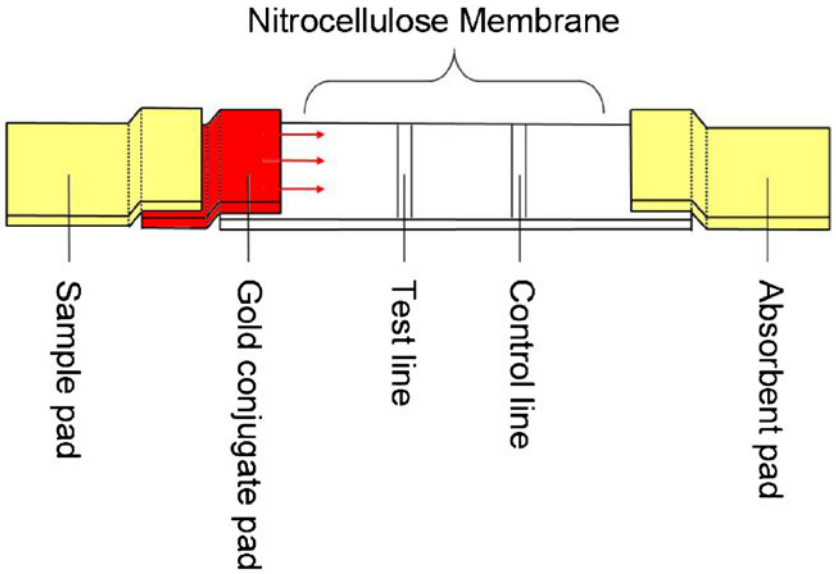

Fig. 1 Schematic diagram of immunochromatographic test strip

Assembling immunochromatographic strip

The test strip was made of three pads: sample, conjugate and absorbing pad and a nitrocellulose membrane (NCM) containing test and control lines (1:3, 1:100 diluted in phosphate buffer solution) (Fig. 1). The pads together with the membranes were all pasted to a semi-rigid polyethylene sheet in a sequence, NCM first, followed by absorbent pad overlapping it by 2 mm, then the conjugate pad from below overlapping the NCM by the same width and finally sample pad overlapping the conjugate pad. The assembled sheet was then cut into strips of 0.37 cm width. The strips were stored sealed in desiccated bags until use.

Sample extraction

One gram of ground corn and rice samples were spiked by standard AFB_1 and AFB_2 (0, 0.5, 2 and 5 ng g^{-1}). Samples were verified by HPLC as uncontaminated. Each sample was gently mixed with a metal spatula, minimizing contact with the walls of centrifuge acid washed tube. The sample was left covered overnight (12–16 h) at room temperature. Two milliliter extraction liquid containing 50:50 (v/v) methanol and Phosphate buffer was added to each sample and further gently stirred with a metal spatula. The mixture was left to stand for only 30 min under room temperature with gentle stirring at 10 min intervals. The mixture was centrifuged at 10000 r/min for 10 min at 25 °C. The clear supernatant was drawn and diluted 10 fold with Phosphate Buffer Solution Tween. In the absence of a centrifuging machine, the mixture was filtered using a filter paper to obtain clear filtrate. The filtrate was diluted 10 fold with phosphate buffer solution with Tween. The diluted solution was then used on the ICG strip (100 μL). To validate our ICG strip's use for other food commodities, the same procedure was applied to barley, peanut, wheat, and soybean. Pure milk was also spiked with a series of AFM_1 standard concentrations 0, 0.1, 0.5, 1, 2, 5 and 10 ng mL^{-1} to validate the developed strip. The milk was stirred gently for a few seconds and then centrifuged at 10000 r/min for 20 min at 4 °C. The clear liquid was diluted 10 fold and used on our ICG strip. Each experiment was subjected to three replications.

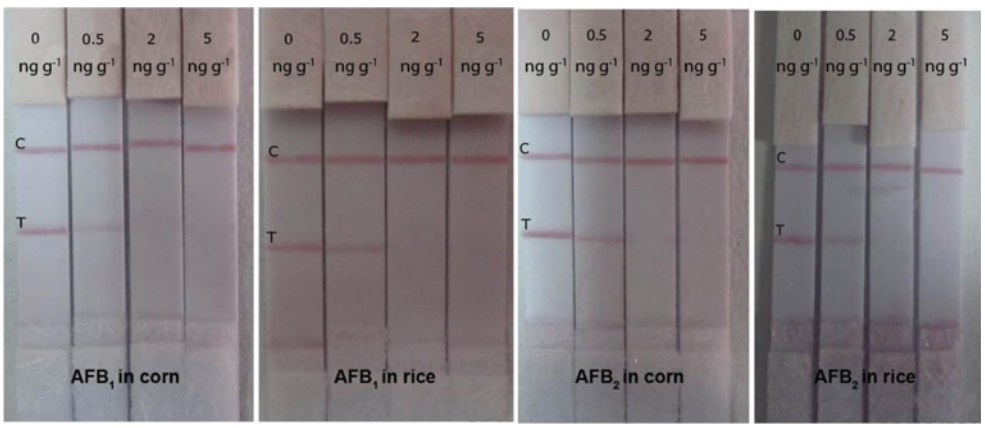

Fig. 2 Detection of AFB_1 and AFB_2 by ICG test strip in spiked corn and rice samples

Table 1 Comparison of recently reported ICG strips with the strip reported in the present paper

Analyte	ICG treatment before use	Indicator label	LOD	Visual detection time	Duration of ICG stability	Food samples tested	References
AFB_1, AFB_2, AFG_1, AFG_2	NCM blocked with PB containing 1% (w/v) BSA; washed twice with PB 0.05% Tween 20; release pads treated with BB1% (w/v) BSA, 0.25% (v/v) Tween 20, 2% (w/v) sucrose and 0.02% (w/v) sodium azide; strips read by scanner connected to PC	colloidal gold	1 μg kg^{-1} and dynamic range 2–40 μg kg^{-1}	10 min	not indicated	corn (maize)	Anfossi et al. 2011 [22]
AFB_1	conjugate and sample pad treated with blocking buffers beforehand	nano-gold-probe	1 and 30 ng mL^{-1}	15 min.	not indicated	peanut, pure-tea, vegetable oil, feedstuff	Zhang et al. 2011 [20]
AFM_1	treated NCM; strips kept refrigerated at 4 °C in plastic bag until use	gold nano particle	1.0 ng mL^{-1}	10 min.	not indicated	milk	Wang et al. 2011 [23]
AFB_2	NCM blocked with BSA (2.0%, w/v) in Phosphate Buffer Solution	magnetic nano-gold microspheres	0.9 ng mL^{-1}	15 min	not indicated	peanut, hazelnut, pistachio, almond	Tang et al. 2009 [21]
AFB_1	treated sample and absorbent pads	nano-colloidal gold	0.5 ng mL^{-1}	15 min	not indicated	rice, barley, feed samples	Shim et al. 2007 [24]
AFB_1	6 h stirring for antibody adhesion to nanoparticles; conjugate pad blocked with PBS-Tween-BSA; NCM blocked with BSA-PBS for 1h	silver core and goldshell (AgAu)	visual detection limit of 0.1ng mL^{-1}	<15 min	22 days at 4°C	rice, wheat, sunflower, cotton, chillies, almonds	Liao & Li 2010 [18]
AFB_1, AFB_2, AFG_1, AFG_2	membrane blocked with 2% (w/v) OVA; sample and conjugate pads treated with blocking buffers and dried at 37 C overnight	nano gold probe	0.03, 0.06, 0.12, and 0.25 ng mL^{-1},	after 15 min	not indicated	peanut	Zhang et al. 2010 [16]
AFB_1, AFB_2 AFG_1, AFG_2 AFM_1	no strip treatment	colloidal gold	0.1ng mL^{-1}	<5 min	6 months at room temperature (15–30 °C)	corn, rice, wheat, barley, soybean, peanut, milk	[present paper]

For real samples, Kenyan corn and rice, the aforementioned procedure for cereals was followed except that there was no spiking. Briefly, 2 mL extraction liquid containing 50:50 (v/v) methanol and Phosphate Buffer was added to each sample of 1 g and gently stirred with a metal spatula. The mixture was left to stand for only 30 min under room temperature with gentle stirring at 10 min intervals. The mixture was either centrifuged at 10000 r/min for 10 min at 25 °C or filtered using filter paper to obtain clear supernatant or filtrate which was then diluted and used on our strip. A sample was identified as positive if it contained either aflatoxin B_1 or B_2 and negative if none of the two were present or were below the detection limit.

Results and discussion

Development of ICG strip

Strip development can be complex due to many factors such as flow properties, matrix effects, blocking buffers, pore size among other challenges [13]. We developed an ICG test strip that is fast, simple and relatively affordable than similar strips developed recently. 100 μL supernatant of cereal or milk was applied directly to the sample well and allowed to migrate along the membrane. The negative test (without AFB_1 or AFB_2) resulted in two red lines in the test and control region lines (C, T). Positive samples gave only one red line in the control region (C). For our ICG strip, it was important that the

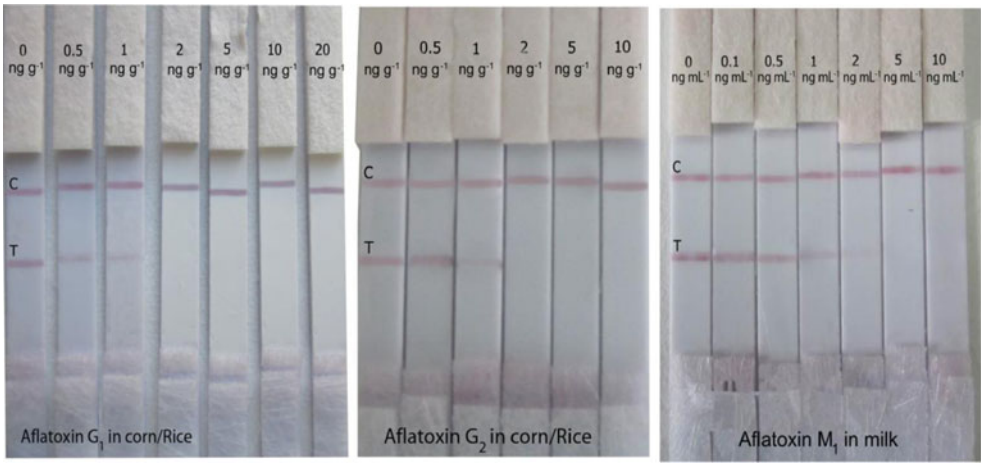

Fig. 3 Cross reactivity of the ICG strip with AFG_1, AFG_2, and AFM_1

color intensity of the test line (T) be strong enough to give a clear visual distinction between negative and positive tests. A faint line in the T-zone ± denoted a warning sign. We therefore selected 2 ng mL^{-1} as our limit of detection in grain samples (Fig. 2, Fig. S1 in ESM). Unlike similar strips (Table 1), our strip can give visual results within a record of 5 min compared to other strips whose visual detection is 10 min and above. Whereas other strips (Table 1) had either the NCM pads, conjugate pads, sample pads or all the pads treated with blocking buffers or modified to improve sensitivity before use [16, 18, 20–24], in contrast our ICG strip did not require any pre-treatment or additional sensors. In addition, the other ICG strips have been validated for just one or a few food stuffs; our ICG strip can be used in six grains (corn, rice, barley, peanut, wheat, soybean) and milk with no variation in pad treatment with an added advantage of the strips conveniently stored in sealed desiccated bags at room temperature. Strip stability was assessed after 1, 2, 3, 4, and 6 months. There was high cross reactivity for AFG_1 and AFG_2 (0.1 ng g^{-1}) in cereals and AFM_1 (0.5 ng mL^{-1}) in milk (Fig. 3). Our ICG strip is therefore one of the simplest, cheapest, versatile and more stable than the others reported recently.

Use of centrifuge and filter paper

Whereas centrifuging was quicker, we opted to use filter paper as well. The results of both the centrifuged supernatant and filtrate from filter paper were identical (Fig. S2 in ESM). This we found to be an added advantage for developing countries which may lack laboratory centrifuges, portable centrifuges or lack electricity to run the same. We therefore concluded that, in the absence of a centrifuging machine, the samples can easily be filtered.

Fugacity theory

Sample preparations and matrix interference remain a considerable source of error in the analytical identification of aflatoxins using lateral flow tests. Thus, systematic approaches to sample preparation and analysis are absolutely necessary to determine aflatoxins at the parts-per-billion level [15, 25, 26]. During extraction and recovery we were cognizant of fugacity theory to minimize matrix effects by reducing the forces that bind aflatoxin to cereals or containers used. The concept of fugacity states that, when a chemical achieves equilibrium

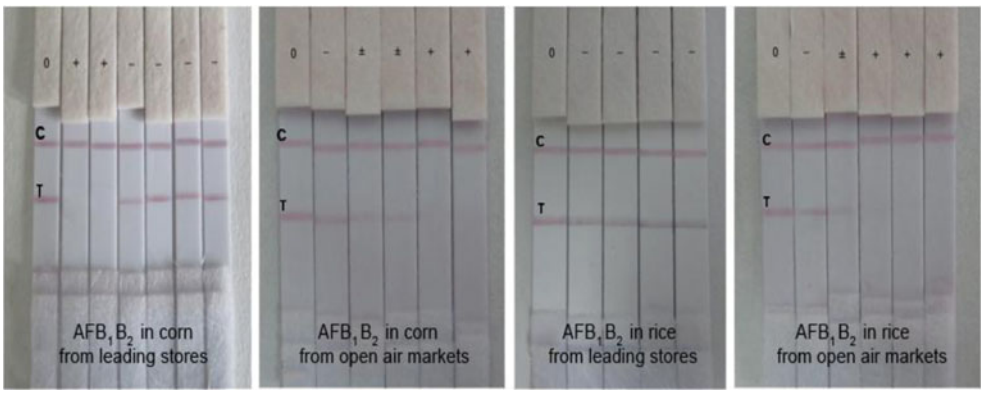

Fig. 4 Detection of AFB_1 and AFB_2 in naturally contaminated Kenyan corn and rice samples

Table 2 Aflatoxin contamination in Kenyan samples

Source of sample	Total samples	+ve samples	-ve samples	%+ve samples
Leading stores				
Rice	27	4	23	14.8%
Corn	26	9	17	34%
Open air markets				
Rice	33	12	21	36%
Corn	27	18	9	66%
Total	113	43	70	38%

between different phases such as liquid, air and organic matter, the fugacities (pressures) are equal in each phase but the concentrations can differ by many orders of magnitude, indicated by the relevant Z-factor indicating phase effects on concentration [27]. It is usually assumed that aflatoxin or any other chemical will be automatically concentrated in the extraction solutions, however if the required equilibrium predicated for fugacity is not achieved, then the solution may lack the desired quantities and concentrations. Many conventional methods apply robust shaking with high volumes of liquids for long periods during extraction. We used fugacity theory to overcome matrix effects that could result from too robust shaking and tilting of tubes during extraction. Too much shaking does not only make the cereal samples finer and difficult to centrifuge, but also may create adhesion between aflatoxin and sample particles or surfaces. This force becomes difficult to break and release the aflatoxin in the liquid during centrifuge or filtration. In our experiment however, we applied short times of gentle stirring of the mixture with a metal spatula (1 min each time), making sure that the sample is not distributed on the walls of the tube; 2 mL of extraction solution, just enough to dilute the sample was preferred to high liquid content above the sample, which may reduce the concentration in the liquid. The mixture was left to stand for 30 min only, as too long extraction time incorporates air in the samples therefore reducing the concentration due to fugacity pressures, possibly increasing matrix effects as the starch disperses or forms glue. During optimization process of our method, we experienced that, too long extraction periods, high volumes of extraction liquid and high speed shaking, all reduced aflatoxin recovery, presumably reflecting physical changes in the extraction system. Phosphate Buffer Solution Tween was chosen as a diluting buffer for it has a surfactant that would break the force of gravity holding aflatoxin to any sample particles in the supernatant. The use of other buffers did not give as good clear results as Phosphate Buffer Solution Tween. Unlike the approach by Lee et al. [25] where each sample had a different treatment to reduce the matrix effects for ELISA, our approach applied the same treatment to all samples resulting in reduced matrix effects that interfere with results (Fig. S3 in ESM). We therefore concluded that understanding fugacity theory could provide answers to matrix interferences.

Sample analysis and validation by HPLC

Corn and rice samples were analyzed by ICG test strip (Fig. 4) and the results confirmed by HPLC. Out of 113 naturally contaminated rice and corn samples, 43 (38%) showed positive aflatoxin contamination and 70 (62%) were negative (Table 2). These results should draw public health concern given that corn product ('ugali') and rice are consumed on a daily basis by about 90% of Nairobians and the country at large [6, 9]. In general, corn had high contamination than rice with 50% chance of buying contaminated corn flour whereas one has a chance of 26% of buying contaminated rice in Nairobi. This difference in levels of contamination between corn and rice can be explained by the fact that corn is highly colonized by aflatoxin producing *Aspergillus* spp.; besides, it is grown in climates that are likely to have perennial contamination with aflatoxins [10, 24, 25]. Rice on the other hand is grown in wetlands, though studies done in India and elsewhere have shown aflatoxin contamination in rice [28].

Open air markets carried more aflatoxin contaminated corn flour and rice than the leading stores. The probability of buying contaminated rice from leading stores was 14.8% while that of buying contaminated rice from open air markets was 36% (over twice as risky). For corn flour, one had a higher chance of getting contaminated corn flour from open air markets (66%) than from leading stores (34%). It has been shown by other studies that informal markets in sub-Saharan Africa support spoilage due to weather changes; abrupt rainfall that wet the grains among other malpractices [9, 10, 29].

Representative samples (Table 3) of each category of ground corn and rice that tested negative or positive using ICG were sent to Tianjin High Standard Quality Testing Laboratory (www.tjhsd.com.cn). AFB_1 contamination was higher than AFB_2. The (b) Kibera sample, for example, showed highest AFB_1 (31.79 ng g^{-1}) followed by (a) Kibera sample with (14.96 ng g^{-1}). This can be explained by the fact that AFB_1 is the most common among the four major aflatoxins and that AFB_2 is a dihydroxy derivative of AFB_1 [3, 28]. Both ICG test strip and HPLC test resulted in good correlation.

Table 3 Comparison between ICG strip and HPLC

Sample (n=20)			
Source of sample	Market[a, b] or Brand[c, d]	ICG results	HPLC results
Corn[a] from open air markets	Kibera	+ve	14.96 ng g^{-1} (B1)
			1.20 ng g^{-1} (B2)
	Kangemi	+ve	7.80 ng g^{-1} (B1)
			0.80 ng g^{-1} (B2)
	Kawangware	-ve	nd (B1)
			nd (B2)
	Gikomba	+ve	7.44 ng g^{-1} (B1)
			0.33 ng g^{-1} (B2)
	Mathare	+ve	3.85 ng g^{-1} (B1)
			nd (B2)
Rice[b] from open air markets	Kibera	+ve	31.79 ng g^{-1}(B1)
			1.67 ng g^{-1} (B2)
	Kangemi	-ve	nd (B1)
			nd (B2)
	Kawangware	-ve	nd (B1)
			nd (B2)
	Gikomba	+ve	2.88 ng g^{-1} (B1)
			nd (B2)
	Mathare	-ve	nd (B1)
			nd (B2)
Corn[c] from leading stores	Soko	+ve	1.86 ng g^{-1} (B1)
			1.20 ng g^{-1} (B2)
	Pembe	-ve	nd (B1)
			nd (B2)
	Incas	+ve	5.51 ng g^{-1} (B1)
			0.50 ng g^{-1}(B2)
	Jimbi	-ve	3.10 ng g^{-1} (B1)
			nd (B2)
	Jogoo	-ve	nd (B1)
			nd (B2)
Rice[d] from leading stores	Rice ex Pakistan	-ve	nd (B1)
			nd (B2)
	Kamili premium	-ve	nd (B1)
			nd (B2)
	Dawat aromatic	-ve	nd (B1)
			nd (B2)
	High fibre rice	-ve	nd (B1)
			nd (B2)
	Pearl pishori	-ve	nd (B1)
			nd (B2)

[a, b]indicate different open air markets - rice and corn was not branded.

[c,d]indicate different brands of rice and corn in leading stores

nd not detactable indicate the concentration is lower than the limit of detection by HPLC

Conclusion

We have developed an ICG strip test suitable for screening the range of aflatoxins in human food and animal feeds that is simple (simpler than similar ICG), fast, affordable, stable and sensitive, versatile and portable. We report for the first time the use of ICG strip in determination of aflatoxin in Kenya. We also report for the first time the screening of rice in Kenya for aflatoxin. To the best of our knowledge, this is one of the simplest ICG strip developed to determine aflatoxin contamination in food commodities. The results of this study should be able to quicken aflatoxin detection, and help in the rapid risk assessment, and hopefully reduce the exposure and fatality rates. The ICG strip could have wide

application in the detection of major aflatoxins and their metabolites.

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