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Determination of aflatoxin M_1 in urine samples indicates frequent dietary exposure to aflatoxin B_1 in the Bangladeshi population

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ABSTRACT

Aflatoxin B_1 (AFB_1), a hepatocarcinogen and highly toxic mycotoxin, is a contaminant of food commodities, especially in hot and humid climates that favour the growth of aflatoxin-producing fungi. As data on AFB_1 contamination of food and feed in Bangladesh are scarce, we conducted an initial screening by ELISA on the occurrence of the metabolite and biomarker aflatoxin M_1 (AFM_1) in urines from Bangladesh which indicated frequent exposure. This finding led us to conduct a follow-up study where we applied a more sensitive method (IAC clean-up and HPLC-FD analysis) to determine AFM_1 concentrations in a larger set of urine samples. To account for possible seasonal and regional differences in mycotoxin exposure, in total 218 urines were collected in two districts of Bangladesh: 164 urines (n = 69 in summer, n = 95 in winter) from residents of a rural and an urban area in Rajshahi district, among them 62 participants enrolled in both sampling periods, and 54 urine samples obtained from pregnant women in Dhaka district.

AFM_1 was detected in >40% of all Rajshahi urine samples at a range of 1.7–104 pg/mL in summer and at a range of 1.8–190 pg/mL in winter season. The mean level of urinary AFM_1 was higher in winter (27.7 ± 42.6 pg/mL) than in summer (13.6 ± 21.2 pg/mL) season, and differences were observed at the mean AFM_1 level between the rural and the urban Rajshahi cohort. AFM_1 was found less frequently in the Dhaka pregnant women (31% above LOD, mean 13.9 ± 33.3 pg/mL), but in a similar concentration range (1.7–141 pg/mL) as in the Rajshahi cohort. Urinary AFM_1 levels did not show significant associations with the participants food consumption pattern. In conclusion, when compared to biomarker data from other countries, detection frequency and urinary AFM_1 levels in our Bangladeshi cohorts raise concerns regarding their exposure to potent carcinogenic aflatoxins.

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1. Introduction

Aflatoxins are highly toxic secondary fungal metabolites produced by *Aspergillus* species (mainly *A. flavus* and *A. parasiticus*) that contaminate crops and dietary staples, including maize and groundnuts (IARC 1993, 2002). Human exposure to these mycotoxins is a serious problem in developing countries where hot and humid climate favours fungal growth and where food storage conditions are poor (Wild and Gong, 2010). Aflatoxin B_1 (AFB_1), the most toxic and most prevalent aflatoxin contaminant in food commodities, is a major cause of disease in parts of Africa and Southeast Asia (Groopman et al., 2008; Wild and Turner, 2002; Williams et al., 2004): It is a significant risk factor for hepatocellular carcinoma, along with hepatitis B virus infection (IARC, 2002; Sun et al., 2013). In addition, aflatoxin exposure early in life can result in impaired child growth (Gong et al., 2004; Turner, 2013), and may modify immune function (Turner et al., 2003). Furthermore, several outbreaks of acute aflatoxicosis have been reported, with the most severe outbreak in 2004 in Kenya (Azziz-Baumgartner et al., 2005).

Biomarkers of exposure to AFB_1 were essential in epidemiological studies that examined the relation between mycotoxin exposure and risk of disease (see reviews by Kensler et al., 2011; Turner et al., 2012; Wogan et al., 2012). Such investigations have determined levels of the serum albumin AFB_1-lysin adduct in human blood whilst others have analysed AFB1-N7-guanine (released from AFB_1- DNA adduct) or the aflatoxin M_1 (AFM_1) metabolite levels in urine. As the concentration of all these analytes are strongly correlated with aflatoxin intake in chronically exposed individuals they can serve as valid exposure biomarkers (Gan et al., 1988; Turner, 2013). Thus, availability of human speci-

Abbreviations: AFB_1, aflatoxin B_1; AFM_1, aflatoxin M_1.

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men (blood or urine) and analytical resources will affect the choice of a suitable biomarker. Blood sampling is invasive and requires medical personal, whilst non-invasive sampling of body fluids (e.g. urine or breast milk) is easier to perform in field studies and often better accepted by the participants. Thus, in general urine is the most widely chosen matrix for biomarker analysis.

Analysis of biomarkers in human body fluids covers mycotoxin intake from all dietary sources and exposure by various routes (Degen, 2011; Fromme et al., 2016). Thus biomonitoring provides valuable insights, especially in developing countries as Bangladesh where AFB_1 food contaminant data are scarce since no regular surveillance of mycotoxins exists (Islam and Hoque, 2013). Bangladesh has a tropical monsoon climate, characterized by seasonal variations in rainfall, high temperatures and high humidity. Three seasons are usually recognized: a hot, humid summer, a rainy monsoon season, and a warm-hot, dry winter (http://en.wikipedia.org/wiki/Geography_of_Bangladesh). Agriculture in Bangladesh is heavily dependent on the weather, and crops are mainly produced in summer and in winter. The harvest of one season is largely consumed in the next season until new seasonal crops become available (Ali, 2016). High humidity at harvest and in storage favours growth of mycotoxin producing moulds on agricultural products. Thus, it is no surprise that occurrence of aflatoxins was reported in plant-derived food (maize, cereals and groundnuts) and feed in Bangladesh (Dawlatana et al., 2002). A more recent study in Bangladesh reported the presence of AFB_1 in pooled samples of eight commonly consumed food commodities and in poultry feed, with 5 of 8 food commodities exceeding the EU regulatory limits for aflatoxins (Roy et al., 2013). The authors discuss a number of limitations in their study, for example seasonal variation in contamination not captured and food types which were not analyzed, and then recommend biomonitoring as complementary approach to further investigate aflatoxin exposure in the Bangladeshi population.

This approach has been applied so far in cohorts from different regions of the country. The presence of AFB_1-lysine adduct in serum has been investigated in 63 women in a rural region in the Northwest of Bangladesh (Gaibandha district): All blood samples taken in their 1st and 3rd trimester of pregnancy were positive as well as cord blood and serum samples collected later from their children (Groopman et al., 2014). The study carried out between 2008 and 2012 indicated rather high AFB_1 exposures in this cohort. In a more recent study, urines collected in 2014 from adult residents of a rural (n = 52) and an urban (n = 43) area in the Rajshahi district were analyzed for the presence of AFM_1 (Ali et al., 2016a): The results of screening for this biomarker by ELISA indicated frequent AFB_1 exposure of these Bangladeshi cohorts. Yet, our initial study has some limitations regarding urine sampling only in one season and district, and the method sensitivity. The present study aim was to develop a more sensitive HPLC-FD method and apply it to an analysis of urinary AFM_1 in a larger set of samples by adding a cohort from a region in central Bangladesh (Dhaka district) and investigating possible seasonal and regional differences in biomarker levels.

2. Materials and methods

2.1. Standards, chemicals and reagents

Methanol (HPLC grade) and acetonitrile were purchased from Promochem (Wesel, Germany). AFM_1 standard solution was from Sigma-Aldrich Chemie (Taufkirchen, Germany). Working standard solutions were prepared weekly by dilutions in mobile phase acetonitrile/water (25:75, v/v). IAC columns, *i.e.* AflaTest® WB^{SR} and $AflaM_1$ HPLC, for enrichment of AFM_1 from urine were purchased from Ruttmann, Hamburg, Germany. The aflatoxin M_1 ELISA kits for analysis in urine samples were purchased from Helica Biosystems Inc., Santa Ana, CA 92704, USA. Information on cross-reactivity of IAC columns for enrichment or the ELISA kit for AFM_1 is limited: The supplier of IAC columns (VICAM) used stated that the AflaM1 column cross-reacts with AFB_1, B_2, G_1, G_2 and AFM_2 (no % given). A recent methodological study by Schwartzbord et al. (2016a) found notable cross-reactivity between the antibody of the Helica ELISA assay for AFM_1 and other aflatoxins: the nominal amounts of "AFM1" were 1.94, 1.01, 0.994, and <0.200 ng/ml in urine samples spiked with 1.0 ng/ml of AFB_1, AFB_2, AFG_1, and AFG_2, respectively.

2.2. Sampling areas and study populations

In total, 218 urine samples were collected from inhabitants of Rajshahi and Dhaka district of Bangladesh (see Fig. 1 for sampling sites). In Rajshahi district, urines were collected in two seasons (n = 69 in summer, May 2013 and n = 95 in winter, February 2014) from adult residents of a rural (Mongol Para, Puthia) and an urban area (Rajshahi University region); among these were 62 participants enrolled in both sampling periods. In Dhaka district, urine samples (n = 54, winter: February–March 2014) were obtained from pregnant women in a rural and a suburban area of Savar region in Dhaka district. All urine donors were informed about the study and a written consent was obtained from them prior to inclusion in our study on AFM_1 and on other mycotoxin biomarkers (Ali et al., 2015, 2016b, 2016c). All participants were of good health and they were asked to fill out a questionnaire for baseline anthropometric information, occupation and daily, weekly and regular food habits. Morning urine samples (approximately 50 mL) were collected in a disposable container from each volunteer and first stored at −20 °C at Rajshahi University and sent on dry ice to IfADo, Dortmund for subsequent analysis. Urinary creatinine was measured by a modified Jaffe method (Blaszkewicz and Liesenhoff-Henze, 2012) to account for differences in urine dilution between individual spot urines. The Institute of Biological Sciences of Rajshahi University, Bangladesh and the institutional Internal Review Board of IfADo approved the study.

2.3. Sample preparation

In brief, urine samples were thawed and centrifuged at room temperature at 3500 rpm for 10 min. Then 10 mL aliquots of urine were adjusted with 1N hydrochloric acid or 1 M sodium hydroxide to keep the pH between 5.5 to 7.0. The sample was then loaded on AflaTest® WB^{SR} at a flow rate of 1 drop/s. Also $AflaM_1$ HPLC columns were used for a number of urine samples (n = 20) to compare the recoveries with AflaTest® WB^{SR}. Columns were washed twice with 10 mL of distilled H_2O, then AFM_1 was eluted (flow rate 1 drop/s) with 2 mL of methanol. Eluates were evaporated to dryness under a stream of nitrogen at 45 °C, and the residue was dissolved in 500 μL of acetonitrile/water (25:75), vortexed and centrifuged at 12000 rpm for 3 min prior to HPLC-FD analysis. Thus the analyte enrichment factor was 20. Details on the ELISA analysis of AFM_1 in urines have been provided previously (Ali et al., 2016a).

2.4. HPLC analysis with fluorescence detection

AFM_1 analyses were carried out with a HPLC Shimadzu system consisting of two LC-10AS pumps, RF-10Axl fluorescence detector, SIL-10AD Vp auto injector, CBM-20A communication module, and Shimadzu LC solution software. A C_{18} Microsorb-MV100 column (150 × 4.6 mm, 5 μm, from Agilent Technologies, Waldbronn, Germany) fitted with a C_{18} Metaguard column (10 × 4.6 mm, Microsorb A104MG) was used at a column temperature of 25 °C, and chromatographic separation of the analytes was achieved by

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