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Preliminary study on the relationship between aflatoxin-bovine serum albumin adducts in blood and aflatoxin M1 levels in milk of dairy cows

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27 would like to thank Christopher Wild (IARC, Lyon, France) for providing the aflatoxin-Cl_2-
28 BSA polyclonal antibody. It was the essential substance for our study.

29

Abstract

The aflatoxin (AF) albumin adduct is often used as a biomarker for aflatoxin exposure in humans. An ELISA method previously used for aflatoxin serum albumin in human blood was used to analyse bovine serum samples (n=22) collected from dairy cattle during an aflatoxin mitigation study in Kenya. Albumin adduct data were compared with aflatoxin M_1 (AFM_1) levels in corresponding milk samples from these cows. The concentration ranged from < LOD to 487.9 pg/mL for AFM_1 and < LOD and 96.3 pg/mg for aflatoxin albumin. This study indicates that aflatoxin albumin adducts could be used as a measure of chronic aflatoxin exposure in dairy cattle.

Keywords: Aflatoxin albumin adduct, biomarker, aflatoxin M_1, ELISA, dairy cow

Introduction

Aflatoxin B_1 (AFB_1) is the most potent mycotoxin for both humans and animals, and is recognised as a human liver carcinogen (IARC, 2002). The metabolism of AFB_1 to the biologically reactive AFB_1-8,9-epoxide leads to the formation of mutagenic DNA adducts, which are key to the carcinogenic process (Eaton et al. 1994). In addition, the epoxide can be hydrolyzed to form AFB_1-dialdehyde, which reacts with the amino group of lysine (ε-position) in serum albumin to yield the aflatoxin albumin adduct in blood (Guengerich et al. 2002; Sabbioni 1990). Hydroxylation of AFB_1 in the liver (Dohnal et al. 2014), yields AFM_1, which is excreted in urine and milk of humans and dairy animals. Although AFM_1 is much less potent than AFB_1, exposure of breastfed infants or dietary exposure from cow milk is another source of aflatoxin exposure. Both aflatoxin-albumin and urinary AFM_1 have been validated as biomarkers against dietary intake of AFB_1 in human populations from high exposure regions (Wild et al, 1992, Routledge et al, 2014, Chen et al, 2017). Aflatoxin-

albumin is seen as a marker of more chronic exposure due to the long half-life time of the albumin in blood, whilst AFM_1 in urine is useful as a marker of very recent exposure. Consequently, aflatoxin-albumin has been used as a biomarker of human aflatoxin exposure in many studies (Turner et al. 2012; Xu et al. 2018). As aflatoxin human serum albumin (AF-HSA) has been useful at measuring chronic exposure in humans we have tested whether aflatoxin bovine serum albumin (AF-BSA) would be useful in measuring chronic exposure in cattle. While AFM_1 residues in milk are not detectable four to five days after toxin administration (Applebaum and Marth 1981; Chopra et al. 1999), AF-BSA could be still detectable due to the longer half-life of albumin (20.7 ± 1.1 days). To our best knowledge, the analysis of AF-BSA in bovine blood was not performed before.

In many of the studies of human aflatoxin exposure, an ELISA developed by Chapot and Wild (1992) has been used to detect AF-HSA. This ELISA uses a rabbit polyclonal antibody that was raised against Aflatoxin-Cl_2-BSA and shows reproducible reactivity with aflatoxin-albumin. Here, we have used this assay to measure AF-BSA in bovine serum samples.

Material and Methods

Sample collection

Farms for sampling dairy cows were recruited from an ongoing trial on aflatoxin mitigation in Kenya. All participating farmers were briefed about the purpose of the project and gave written informed consent. The farms were located in Kasarani, in peri-urban Nairobi, where smallholder farmers hold an average of six dairy animals, almost exclusively exotic breeds or crossbreeds, and producing on average a total of 27 litres of milk per day per farm (Kagera et al. 2019). The animal sampling had ethical approval from the Institutional Animal Care and Use Committee (IACUC) at the International Livestock Research Institute (IACUC 2017.03). At the time of sampling, blood was collected from a jugular vein of the cow, and a milk

sample was also collected. Samples were kept in a cool box with ice packs to keep the samples as cool as possible during transport. The blood samples were centrifuged and the serum aliquoted. Serum samples were transported to Leeds on dry ice and kept frozen at -20 ^{0}C until analysis.

Analysis of AFM_1 in milk

The analysis of AFM_1 was performed with a commercial enzyme-linked immunosorbent assay (ELISA, Helica Biosystems Inc., Santa Ana, CA, United States, Catalogue No. 961AFLM01M-96) according to the manufacturer's instructions (Senerwa et al. 2016). A detailed evaluation of the ELISA kit was published by our partner (Imtiaz and Yunus, 2019). In brief, thawed milk was centrifuged at 2,000 g for five minutes to induce separation of the upper fatty layer. The upper fatty layer was removed and the lower plasma was used in the assay. Standards and samples (200 µL) were aliquoted to the pre-coated plates in duplicate. After incubation and washing, 100 µL of conjugate was added. After 15 minutes incubation and washing, 100 µL of enzyme substrate was added to each well and incubated for 15 minutes before adding 100 µL of stop solution. The optical density of each microwell was read using a microplate reader at 450 nm and the level of AFM_1 in each well was calculated using a logarithmic standard curve, with the average of the duplicates used as the result. The ELISA used had a lower limit of detection of 2 pg/mL. Samples exceeding the highest standard (100 pg/mL) were diluted and re-tested.

Analysis of aflatoxin-albumin adduct in blood serum

Samples of BSA were analysed for AF-BSA according to the method published by Chapot and Wild (1991) for the analysis of AF-HSA. In outline, this analysis is performed in four steps: albumin extraction and quantification, hydrolysis of albumin with pronase, purification of aflatoxin-albumin residues and the competitive ELISA. The ELISA involves the pre-

106 mixing of standards, samples or controls with the rabbit anti aflatoxin-Cl_2-BSA polyclonal
107 antibody (a gift from Christopher Wild, IARC), followed by direct ELISA in which remaining
108 unbound antibody can bind to aflatoxin ovalbumin on the surface of the ELISA plate well.
109 After washing, the bound primary antibody is detected by incubation with an enzyme labelled
110 goat anti-rabbit secondary antibody. Bovine and human control samples (both blank and
111 spiked with aflatoxin-albumin produced by reaction of the albumin with AFB_1-8,9-epoxide
112 prepared in our laboratory) were examined in each experiment. Additionally, the extractions
113 were repeated using random samples (n=6) in order to ensure reproducibility of data.
114 The specific procedures involved the following steps. An aliquot of 200 µL serum was heated
115 at 56 °C in a water bath and saturated ammonium sulphate (300 µL) was added dropwise after
116 cooling the sample on ice. After centrifugation, the supernatant was removed and adjusted to
117 pH 5 with 1M acetic acid to precipitate the albumin. After another centrifugation, the
118 supernatant was discarded and the albumin pellet was redissolved in phosphate buffered
119 saline (PBS). After that, the extracted albumin was quantified using a BIO-RAD protein assay
120 dye reagent (Bradford-test, BIO-RAD, Watford, UK) and human serum albumin (Sigma,
121 Gillingham, UK) as standard. For that purpose, 40 µL of filtered Bio-rad reagent were
122 pipetted into each well of a 96 well microplate. A volume of 160 µL of samples or protein
123 standards (n=8, calibration range 0 – 30 µg/mL) were added and mixed. All samples and
124 standards were immediately measured in duplicate at 620 nm.
125 An aliquot equivalent to 2 mg albumin was incubated with pronase overnight in a water bath
126 at 37 °C to hydrolyse the albumin. 100 µl BSA (containing 10 mg BSA) was added to exhaust
127 the rest of the pronase and the proteins were precipitated by adding 1.8 mL cold ethanol. The
128 samples were left at -20 ºC for at least two hours. After centrifugation, the supernatant was
129 diluted with PBS to reduce the final ethanol concentration to < 5 %. The extracts were
130 purified with Sep-pak C-18 cartridges (Waters, MO, USA) using an eight-channel peristaltic
131 pump. After loading the hydrolysed sample, the cartridges were washed with distilled water

and 5 % methanol. The aflatoxin-lysine residues were eluted with 80 % methanol, dried overnight and finally reconstituted in 0.5 mL PBS. AF-BSA was determined using a competitive inhibition ELISA with AFB_1-ovalbumin (self-prepared) as coating antigen and AFB_1-lysine (self-prepared) as the inhibitor for standard curve generation. A 96-well plate (high binding, Greiner bio-one, Stonehouse, UK) was coated with 50 µL AFB_1-ovalbumin per well. After drying overnight at 37 °C the plate was washed five times with PBS + 0.05 % Tween 20. Afterwards, a volume of 200 µL/well of freshly prepared 5 % milk solution containing dried skimmed milk powder (Marvel, London, UK) in PBS (pH 7.4) was added and incubated for one hour in the dark at room temperature, followed by washing five times. Samples, controls or standards were premixed with the primary anti-aflatoxin antibody in 1:1 volume and incubated in the dark at room temperature for 30 minutes. Samples etc. were then added to the corresponding wells in the ELISA plate. The plate was incubated for 90 min protected from light and washed five times with PBS Tween 20. Afterwards, the secondary antibody (goat anti-rabbit IgG peroxidase labelled antibody, Sigma, Gillingham, UK) was added. After incubation (90 min in the dark) and washing, 50 µL of TMB peroxidase substrate/well (3,3,5,5,-Tetramethylbenzidine (Sigma, Gillingham, UK) in citrate buffer pH5) were added and incubated at 37 °C for 20 min. To terminate the enzymatic reaction, 50 µL of 1M HCl was added and the absorbance of the yellow product was read at 450 nm.

The detection limit (LOD) was 3 pg AF-lysine equivalents per mg BSA and each ELISA included three positive and one negative control samples for quality control. All samples were measured in duplicate for each ELISA batch and repeated at least two times on separate days to confirm the results. Results were accepted when values within each ELISA had a %CV below 10 % and samples tested on separate occasions had a %CV below 15 %. If samples lay above the linear part of the standard curve the ELISA was repeated at a suitable dilution. For data presentation, the dilution factor as well as a conversion factor of 4.57 (AF-lysine to AF-BSA) was taken into account.

158

159 *Analysis of serum albumin*

160 Serum albumin was directly determined in serum with a photometric measurement system
161 (Eurolyser VET CCA, Salzburg, Austria). The used commercial kit (Greiner Diagnostic
162 GmbH, Bahlingen, Germany) based on the formation of a coloured complex with
163 bromocresol green in an acidic environment. The lower LOD is defined as 0.3 mg/mL.

164

165 *Calculation and statistics*

166 Values under LOD were set to zero before statistical analysis using Statistica 64 (TIBICO
167 Software Inc., Version 13.0, 2017). The data were not normally distributed which was tested
168 with the Shapiro-Wilk test and were therefore expressed as median, minimum and maximum.
169 The relationship between AF-BSA and AFM_1 in milk was analysed by linear regression
170 analysis using Statistica software package.

171

172 **Results and Discussion**

173 Due to the reaction of the metabolised AFB_1-dialdehyde with the amino group of lysine in
174 serum albumin, aflatoxin albumin can be analysed in blood as a biomarker of AFB_1 exposure.
175 Here, we have applied an ELISA method that has been used to assess AF-HSA levels in
176 numerous populations over many years (Gong et al. 2004; Wild et al. 1990; Xu et al. 2018) to
177 the measurement of AF-BSA levels from exposed cattle and compare this biomarker with the
178 well-established biomarker AFM_1 in milk. Although the ELISA has been mainly applied to
179 the measurement of AF-HSA, this method has previously shown a dose response of aflatoxin-
180 albumin in serum from aflatoxin exposed pigs (Meissonnier et al. 2008). In our pilot study,
181 samples from Kenyan cattle (n=22) were selected due to the higher risk of aflatoxin
182 contamination. The serum albumin concentration of each sample was measured (Eurolyser

183 method) as an indicator of animal health status and to exclude any influence of albumin
184 concentration influence on the AF-BSA concentration. The albumin concentration ranged
185 between 27.7 and 35.5 mg/mL with a median of 31.4 mg/mL. The reference range for
186 ruminants is specified as between 30 to 42 mg/mL (Kraft and Dürr 2005), so we conclude that
187 the cows had a healthy status. Slight fluctuations can be justified by different ages or breed of
188 the sampled cows.
189 The analysed AFM_1 concentration in milk ranged between <LOD and 487.9 pg/mL (median
190 60.1 pg/mL, see supplementary material). The AFM_1 concentration in milk is regulated by the
191 European Commission with a maximum limit of 0.05 µg/kg (equivalent to 51 pg/mL;
192 European Commission 2006).Thus, about 60 % of the analysed milk samples exceeded the
193 upper limit of the European Commission. The maximum value was 9.6-fold higher than the
194 set upper limit. These AFM_1 concentrations are in accordance with Kenyan data reported in
195 the year 2009 (Kang'ethe and Lang'a 2009), where over 600 milk samples were analysed
196 (range 5 to 780 pg/mL). Depending on the types and location of farms from which samples
197 were taken, up to 55.6 % of the positive samples in that study exceeded the AFM_1 regulations.
198 Further surveys in Morocco and Ethiopia reported exceedances of the AFM_1 European
199 Commission maximum limit in raw milk samples (El Marnissi et al. 2012; Gizachew et al.
200 2016). However, the occurrence of AFM_1 is a worldwide problem and is not only related to
201 climatic and geographic differences. The various agriculture systems including the global
202 trade of feedstuffs, feeding types and storage conditions can influence the amount of AFM_1
203 (Galvano et al. 1996; Iqbal et al. 2015).
204 The AF-BSA concentration in serum samples from the 22 Kenyan cows ranged between <
205 LOD and 96.3 pg/mg (median 20.3 pg/mg, see supplementary material), with only one sample
206 having an AF-BSA concentration below the detection limit. The AFM_1 concentration in milk
207 of the same cow was also lower than the LOD. Figure 1 shows the datasets of AF-BSA (x-
208 axis) and AFM_1 (y-axis). To get a first impression, a linear regression with prediction

intervals was included. It may be supposed that about 70 % of the variation (coefficient of determination ($r^2 = 0.69$)) of the values can be explained by this relationship. The significant slope of the linear regression between AF-BSA and AFM_1 would suggest that the AFM_1 concentration in milk increases by about 4 pg/mL when the AF-BSA concentration increases in blood by 1 pg/mg.

However, the dataset is very limited and the range above 60 pg/mg BSA or 200 pg AFM_1/mL contains only two samples. If the relationship between biomarker is regarded without these two samples, the coefficient of determination is lowered to 0.33.

Considering the individual animal and analytical variation, it can be assumed that the number of samples is not sufficient to evaluate the relationship of both biomarkers. However, AF-BSA should be investigated further as a marker for chronic aflatoxin exposure relating to animal health. In rats, it was shown that the level of binding to serum albumin reflects the level of AF bound to liver DNA (Wild et al. 1986). Thus, in regions of high exposure, it may be that the concentration of AF-BSA is inversely related to animal health.

In summary, AF-BSA as well as AFM_1 in milk was analysed in 22 bovine samples, which were collected from a trial on aflatoxin mitigation in Kenya. About 60 % of the cows exceeded the maximum limit of AFM_1 in milk of the European Commission, which reflects the present problem regarding aflatoxin in food and feed in African countries. Furthermore, there was a relationship shown between AFM_1 and AF-BSA, albeit from a limited dataset. However, AF-BSA is a chronic marker that should be evaluated further with regard to animal health, especially as climate change may lead to increased occurrence of aflatoxin in animal feeds. Further evaluation of AF-BSA in controlled studies with supplemented data regarding the nutritional and health status of the cows is recommended.

Conflicts of Interest: None.

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List of Figures

Fig 1 Linear relationship between the aflatoxin albumin adduct (AF-BSA) concentration in blood serum and aflatoxin M_1 (AFM_1) concentration in milk of Kenyan dairy cows (n=22, AFM_1 [pg/mL] = -17.54 + 4.24 · AF-BSA [pg/mg]***, $r^2 = 0.69$, $^{***}p < 0{,}001$; and the prediction interval (dashed line, $\alpha = 0.05$)