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VALIDATION AND ANALYSIS OF AFLATOXIN B1 BY INDIRECT COMPETITIVE ENZYME-LINKED IMMUNOSORBENT ASSAY IN URINE SAMPLES FROM CHILDREN IN LONDRINA, PARANÁ STATE, BRAZIL

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ABSTRACT

Aflatoxins (AFs), secondary metabolites produced by fungi of the genus Aspergillus spp., are recognized worldwide as a public health problem due to their potent carcinogenic, hepatotoxic, and immunosuppressive effects. Aflatoxin contamination in children is of particular concern given the negative impacts on health, development, and quality of life. The objective of this study was to validate the indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) for the analysis of aflatoxin B1 (AFB1) in urine, as well as to evaluate the level of this mycotoxin in 150 urine samples from children living in Londrina, Paraná State, Brazil. The analytical performance parameters evaluated in the validation included specificity (matrix interference and comparison of curves with and without matrix), accuracy (recovery test), precision (repeatability and intermediate precision), robustness, sensitivity, linearity, limit of detection (LD) and limit of quantification (LQ). In the intralaboratory validation, matrix interference was analyzed with dilution factors of 2, 5 and 10 times, and the 5-fold dilution was selected to proceed. The standard curves prepared in the presence and absence of a matrix did not show significant differences in the percentage of linkage between the six points analyzed ($p > 0.05$). The average recovery rate for urine samples contaminated with AFB1 at concentrations of 1.0; 2.5 and 5.0 ng/mL were 100.00 ± 8.71%, 90.00 ± 2.00%, and 93.66 ± 1.52%, respectively. The coefficients of variation of the precision parameters were less than 15%. The regression equation obtained from seven standard curves was y=−14.89ln(x)+61.234y = -14.89 \ln(x) + 61.234y=−14.89ln(x)+61.234, with a coefficient of determination (R^2) greater than 0.99. The LD was 0.096 ng/mL and the LQ was 0.115 ng/mL. AFB1 was detected in 39.3% of the samples analyzed, with concentrations ranging from 0.13 to 9.40 ng/mL and an average of 1.63 ± 1.57 ng/mL. Based on the results obtained, the validated ic-ELISA proved to be adequate for the determination of AFB1 in infant urine. Preliminary data suggest that the level of aflatoxin contamination in children in the region of Londrina, Paraná, Brazil, is low.

Keywords: Monoclonal Antibodies. Fungi. Mycotoxin. Immunodiagnosis.

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INTRODUCTION

Aflatoxins (AFs) represent a serious problem for public health and the global economy, as demonstrated by their frequent detection in food and agricultural commodities (LIU et al., 2013). This group includes about 20 fungal metabolites, produced mainly by the species *Aspergillus flavus* and *A. parasiticus* (VARGA et al., 2011), and only four of these compounds (B1, B2, G1 and G2) are considered significant food contaminants (BELOGLAZOVA; EREMIN, 2015). Fungal contamination of food occurs mainly during pre-harvest, transport and storage. In this context, aflatoxins have been detected in several agricultural commodities, with a higher incidence in corn, peanuts, cotton seeds and dried fruits (IARC, 2002).

Among the different aflatoxin analogues (AFs), aflatoxin B1 (AFB1) is the most toxic, and the extent of toxicity depends on how the liver is affected. The International Agency for Research on Cancer (IARC) classifies AFB1 in Group 1, that is, as carcinogenic to humans (IARC, 2002). FAs are highly fat-soluble, which facilitates their absorption by both the gastrointestinal tract and the respiratory system. Once in the bloodstream, they are distributed to various tissues and organs (IARC, 2002). To exert its carcinogenic activity, aflatoxin requires metabolic activation to generate an electrophilic intermediate, which can react and modify DNA, resulting in pro-mutagenic lesions that activate proto-oncogenes and inactivate tumor suppressor genes (KENSLER et al., 2003; BBOSA et al., 2013).

In addition to being a potent hepatocarcinogenic, mutagenic and teratogenic, aflatoxin B1 (AFB1) also has immunosuppressive properties (SHARMA et al., 2011). The immunotoxic effects of AFB1 are associated with interference with cell-mediated immunity, resulting in a reduction in the number of circulating lymphocytes, inhibition of lymphocyte blastogenesis in several animal species, alteration of Natural Killer cell activity, modulation of cytokine expression, and possible alteration of macrophage function. In addition, AFB1 impairs the ability of bone marrow cells to form myeloid and erythroid colonies (BIANCO et al., 2012; GREINER et al., 2013).

Aflatoxicosis, poisoning caused by the ingestion of aflatoxins (AFs), can affect humans and other animals, manifesting itself acutely or chronically. Cases of acute aflatoxicosis in humans have already been recorded in populations of developing countries, especially in Africa and Asia (MWANDA, OTIENO, OMONGE, 2005; SAMUEL et al., 2009; FAO, 2014). Several factors can influence toxicity, such as environmental

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conditions, age, health and nutritional status, as well as the dose and duration of mycotoxin exposure (FDA, 2016).

Children, due to the still developing immune and metabolic systems, are particularly susceptible to the toxic effects of aflatoxins (AFs). Among the main impacts are the hepatotoxic effects, which can cause severe liver damage and increase the risk of liver cancer. Prolonged exposure to FAs can lead to immunosuppression, leaving children more prone to infections. In addition, chronic exposure can interfere with physical growth and impair cognitive development, compromising learning and school performance (ALVITO, SILVA, 2022; DAI et al., 2024).

Based on this context, the present study aimed to standardize and validate, in the laboratory, the indirect competitive ELISA method for the detection of AFB1 in infant urine samples. The study also sought to evaluate the levels of aflatoxin contamination in children living in Londrina, Paraná State, Brazil. The choice of urine as an analysis matrix aims to facilitate the adherence of the participants, ensuring greater acceptance by parents and children.

MATERIAL AND METHODS

AFLATOXIN B1 PATTERN

The AFB1 pattern obtained from *Aspergillus flavus* (A663, Sigma, USA) was quantified in a spectrophotometer (Biochrom, Libra S22 model, UK), according to the Adolfo Lutz Institute (IAL, 2008). The molar absorptivity of AFB1 considered for the calculation of the concentration of AFB1 in methanol was 21,800 at 360 nm.

URINE SHOWS

A total of 150 children, <1 year old (38) and 1-8 years old (113) participated in this study with the permission of their guardians. The volunteers were informed about the study protocol, and an informed consent form was signed. Urine samples were obtained at the Municipal Laboratory of Children's Emergency Care, Londrina, using a disposable sterile infant cup, followed by storage in a -20°C freezer until the time of analysis. The project was approved by the Ethics Committee for Research Involving Human Beings (CEP-UEL, CAAE 06371612.5.0000.5231).

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CULTIVATION AND PURIFICATION OF HIBRIDOMA AF.4

The hybridoma AF.4 lineage, secretor of monoclonal antibody (AcM), is derived from myeloma strainage Sp2/0-Ag14 and splenic BALB/c mouse cell (KAWAMURA et al., 1988). The hybridoma shows cross-reactivity with AFB1 of 100%, AFB2 of 2.3%, AFG1 of 3.4%, AFG2 of 2.4%, and AFM1 of 4.5%. Culture was initially carried out in RPMI 1640 medium (Gibco Co., USA) plus 50% of Fetal Bovine Serum (SFB, Gibco Co., USA), with gradual reduction of FBS up to 10% concentration throughout cell culture. Followed by increasing gradual adaptation to Hybridoma Serum Free Medium (H-SFM, Gibco, USA) at 37°C, 5% CO2 (Scientific Form, USA). The produced supernatant was filtered, and the AcM was purified by precipitation with ammonium sulfate (243g/L). After purification, the antibody was dialyzed against 1L of PBS at 4°C for 16h (4x), and stored at -80°C. Figure 1 shows the steps performed for cell culture and purification of AcM.

INDIRECT COMPETITIVE ELISA

Determination of AFB1 in urine by indirect competitive ELISA

Determination of AFB1 was performed according to Kawamura *et al.* (1988). Microplate was coated with 50 L of OTA-BSA (250 ng/mL, Sigma) in 0.015M PBS buffer pH7.3 and incubated for 18 h at 4º C. After three washes with 0.05% PBST (PBS+ 0.05% of Tween 20), the wells were blocked with 150 L of bovine albumin solution (BSA, Sigma, USA) 0.1% in PBS and the microplate incubated for 1 h at 25 ºC. After three washes with PBST 0.05 %, 50 L of standard AFB1 or urine and 50 L of anti-AFB1 ACM1 (1:5000, 239 ng/mL) in PBST were added. After incubation at 25°C for 1 hour, the microplate was washed three times with 0.05 % PBST and 50 L of IgG-HRP conjugate (Sigma) diluted in 0.05 % PBST (1:5000) was added, incubated for 1 h at 25ºC. After six washes with 0.05% PBST, 100 L of tetramethylbenzidine chromogen substrate was added, followed by incubation at 15 min at 25ºC. The reaction was blocked with 50 L of H2SO4 1mol $^{L-1}$ and the absorbance reading was performed at 450 nm (Biochrom, Libra S22 model, UK, Figure 2). The mean absorbance was calculated from the triplicate measurement of each concentration and the result was expressed as a percentage of binding by Equation **(1):**

% of binding = $(A+/A^{-}) \times 100$ **(1)**

A+ is the absorbance in the presence of a sample or AFB1 (standard) and A^{-} is the absorbance in the absence of AFB1. The concentration of AFB1 in the samples was

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determined using the standard curve of AFB1 (1.0 to 10 ng/mL), by plotting the percentage of binding against the log of the concentration of AFB1.

Intralaboratory validation of IC-ELISA for quantification of AFB1

The parameters for intralaboratory validation of AFB1 in urine by ic-ELISA were based on linearity, sensitivity, specificity, accuracy, precision, robustness, limit of detection (LD) and limit of quantification (LQ) (Table 1). To validate the methodology, a *pool* of urine samples previously centrifuged at 10,000 rpm for 30 seconds (Chibitan II, Tokyo, Japan) was used (ANVISA, 2003; EC, 2006; INMETRO, 2007).

Table 1. Parameters of intralaboratory validation of AFB1 in infant urine by ic-ELISA

Validation parameters	Tests run
Sensitivity	Slope of the linear regression line
Linearity	Linear regression equation
Specificity	*Matrix interference* Contamination-free matrix (2, 5 and 10x dilution) 3 replicates in 4 different days
	Pattern curve without matrix x matrized pattern curve 3 replicates on 3 different days
Accuracy	*Recovery* 3 determinations on different days, 7 replicates/day 1,0; 2.5 and 5 ng/mL
Precision	*Repeatability* 1 determination, 7 replicates 1,0; 2.5 and 5 ng/mL
	Intermediate accuracy 3 determinations on different days, 7 replicates/day 1,0; 2.5 and 5 ng/mL
Robustness (Sensitivity to small variations)	Awareness time Two different analysts Using a different brand of micropipette
Limit of Detection (LD)	Average - 3.143 x SD Mean and SD: 7 replicates of white
Limit of Quantification (LQ)	Medium - 5 x SD Mean and SD: 7 replicates of white

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Sensitivity

Sensitivity was obtained by the slope of the calibration regression line, according to the equation Sensitivity = response variation / concentration variation (INMETRO, 2007).

Linearity

Linearity was obtained by external standardization according to the equation of the line of seven calibration curves with six points (0.1, 0.2, 0.5, 1.5, 5.0, 10.0 ng of AFB1/mL) (ANVISA, 2003; EC, 2006; INMETRO, 2007).

Specificity

Specificity was evaluated by matrix interference without contamination at 2, 5 and 10x dilutions. In addition, a comparison was made between the points of the standard curve using the standard solubilized in the matrix and methanol:PBST (1:9; v/v). The comparison between the two standard curves was made by the t-test, and the analysis of the deviations between the reference curve and the matrized curve - % of interference (INMETRO, 2007).

Accuracy

Accuracy was evaluated by the recovery test at three levels of contamination (1.0, 2.5 and 5 ng mL), analyzed in three replicates on three different days (EC, 2006; INMETRO, 2007).

Accuracy

Accuracy was evaluated by repeatability and intermediate accuracy. Repeatability was performed by intra-run accuracy, considering three levels of contamination (1.0, 2.5 and 5 ng/mL) analyzed in seven replicates on the same day. Intermediate precision was achieved by inter-race precision, considering three levels of contamination (1.0, 2.5 and 5 ng/mL), analyzed in seven replicates on three different days (ANVISA, 2003; EC, 2006; INMETRO, 2007).

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Robustness

Robustness was evaluated by the execution of the standard curve by two different analysts, variation in the time of plate sensitization (18 and 20h), and use of pipettes of different brands (ANVISA, 2003; EC, 2006; INMETRO, 2007).

Limit of Detection (LD) and Quantification (LQ)

The LD was determined by the formula LD = Mean - (3 x SD) and LQ = Mean - (5 x SD). The mean and SD were obtained from seven replicates of the white of the matrix (INMETRO, 2007).

STATISTICAL ANALYSIS OF THE DATA

All data obtained were expressed as mean and standard deviation of the mean. The data were analyzed for normality and homogeneity, followed by analysis of variance. To evaluate robustness and compare the unmatrized and matrized curves, the t-test was used. Differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

CELL CULTURE

The total volume of supernatant produced in H-SFM medium was 12 liters. The anti-AFB1 AcM precipitated with ammonium sulfate, and the dialysate yielded a volume of 346 mL (508 mg Ac). The dialysate volume was aliquoted at 10 mL and frozen at -20°C. The aliquot used for ic-ELISA was at a concentration of 1.199 mg/mL.

VALIDATION OF THE IC-ELISA

Linearity was evaluated by the calibration curve made with six points of previously quantified AFB1. The regression equation obtained from seven standard curves was $y = -14.89\ln(x) + 61.234$, with a coefficient of determination (R^2) of 0.9936. Table 2 shows the percentage of binding, the mean absorbance, and the coefficient of variation (CV) for each concentration of AFB1 (0.1 to 10.0 ng/mL) used in the preparation of the standard curve. The binding percentage ranged from 26 to 92%, with mean absorbance ranging from 0.24 ±0.02 to 0.87 ±0.07, respectively.

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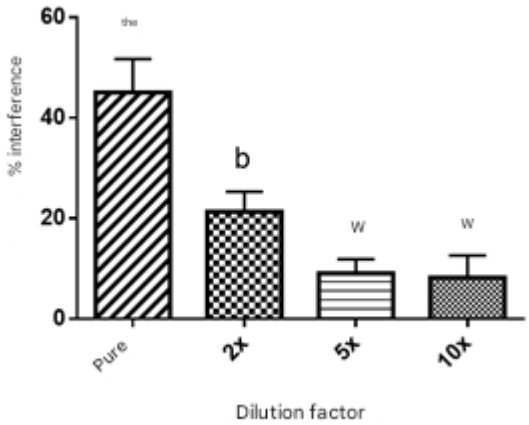
Table 2. Characterization of the points of the aflatoxin B1 standard curve by ic-ELISA

AFB1 (ng/mL)	% link	Average absorbance*	CV (%)
0,1	92,48	0.87 ±0.07	8,34
0,2	86,39	0.81 ± 0.05	6,35
0,5	73,94	0,69 ± 0,06	9,77
1,5	57,05	0,53 ± 0,05	9,81
5,0	35,43	0,33 ±0,02	7,17
10,0	26,40	0,24 ±0,02	9,94

*Values represent the average value of seven standard curves in triplicate performed on different days
CV: coefficient of variation

Specificity was first evaluated by matrix interference diluting the samples at a factor of 2, 5 and 10x (Figure 3). The pure, 2x, 5x and 10x diluted sample showed an interference of 45.1 ± 7.1% (CV = 12%); 21.3 ± 3.63 % (CV = 4%); 9.0 ± 1.23 % (CV = 1%); and 8.0 ± 2.73% (CV = 2%); respectively. Samples diluted in the factor from 5x did not show significant interference ($p < 0.05$) and presented a coefficient of variation within the specified by legislation (CV < 15%; ANVISA, 2003). The 5x dilution factor of the sample was chosen to proceed with the other intralaboratory validation tests.

Figure 3. Matrix interference in ic-ELISA for determination of AFB1 in infant urine. Results expressed as mean % of interference, n = 12. Different letters showed a significant difference ($p < 0.05$).



Once the dilution of the matrix was defined at the 5x factor, the standard curves were compared in the absence of the matrix (AFB1 in PBST:Methanol, 9:1, v/v) and in the presence of the matrix (AFB1 in a pool of urine diluted in the factor of 5x in PBST:Methanol, 9:1, v/v). Figure 4 shows the similarity between the curves made with concentrations of 0.1 to 10 ng of AFB1/mL ($p > 0.05$). The curves prepared without and with the matrix showed VC ranging from 0.7 to 6.5%, and 2.5 to 6.4%, respectively.

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Figure 4. Comparison between standard curve without matrix and matrized curve for determination of AFB1 in urine. The squares represent the average of the standard curve performed in triplicate on three different days.

The limit of detection (LD) and the limit of quantification (LQ) were 0.096 and 0.115 ng/mL, respectively. The recovery test allowed the accuracy of the method to be assessed. The average recovery rate for contaminated infant urine (n = 3) at levels of 1.0; 2.5 and 5.0 ng of AFB1/mL were 100.0 ± 8.71% (CV = 8.7%), 90.0 ± 2.0% (CV = 2.2%), and 93.66 ± 1.52% (CV = 1.6%), respectively (Table 2). At the three concentrations evaluated, recovery rates for AFB1 are within the recommended value of 70 to 110% for concentrations of 1 to 10 ng/mL (EC, 2006).

The accuracy parameters of the method were evaluated for repeatability and intermediate accuracy (Table 3). The average repeatability for contamination is 1.0; 2.5 and 5.0 ng of AFB1/mL were 0.90 ± 0.01; 2.26 ± 0.03 and 4.76 ± 0.01 ng/mL, respectively. For intermediate accuracy, considering the same levels of contamination, the mean was 1.00 ± 0.08; 2.26 ± 0.04 and 4.70 ± 0.07 ng/mL. Both accuracy and precision parameters showed coefficient of variation values lower than 15%, indicating little variation in intra-run and inter-run readings.

Table 3. Accuracy (retrieval) and precision (repeatability and intermediate precision) parameters of ic-ELISA for determination of AFB1 in infant urine

AFB1 (ng/mL)	Recovery		Repeatability		Intermediate accuracy	
	Average (%)	CV (%)	Average (ng/mL)	CV (%)	Average (ng/mL)	CV (%)
1,0	100,0 ± 8,71	8,71	0,90 ± 0,01	2,99	1,00 ± 0,08	8,94
2,5	90,00 ± 2,00	2,22	2,26 ± 0,03	4,70	2,26 ± 0,04	1,95
5,0	93,66 ± 1,52	1,63	4,76 ± 0,01	3,23	4,70 ± 0,07	1,50

CV: coefficient of variation

Robustness was evaluated by the accuracy of the analyses in three variables in the ic-ELISA. The first variable tested was a change of operator to prepare the standard curve at concentrations of 0.1 to 5.0 ng/mL, with a low coefficient of variation of 1.7 to 8.7 %. The result indicated a low variation with the change of analysts. The AFB1 sensitization time for 18 and 20 hours and the use of micropipettes of different brands to perform the ic-ELISA were evaluated by repeatability and intermediate precision. Both parameters showed low coefficient of variation values, as already discussed. Thus, it is possible to say that the method is robust for the three variables tested.

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DETERMINATION OF AFB1 IN INFANT URINE

In this study, we chose to use urine samples due to their advantages over blood collection, especially in the case of children. This choice considered ethical, practical and clinical aspects, highlighting the non-invasive nature of the procedure, which provides greater comfort to children and better acceptance by their guardians. These factors contribute significantly to adherence to studies.

Table 4 shows AFB1 levels in 150 infant urine samples. AFB1 was detected in 39.3% (n = 59) of the samples, with mean levels ranging from 0.13 to 9.40 ng/mL (mean 1.63 ± 1.57). A total of 14 samples (21%) showed contamination ranging from 0.13 to 0.99 ng/mL (mean 0.62 ± 0.28), and only 12 samples (8%) showed a range from 0.13 to 9.4 ng/mL (1.63 ± 1.57).

Table 4. Distribution of AFB1 levels in naturally contaminated infant urine of children from Londrina, Paraná

AFB1 (ng/mL)	n	Positive Displays (%)	Variation (ng/mL)	Average (ng/mL)
n.a.	91	-	-	-
>0,096 – 0,99	21	14	0,13 - 0,99	0,62 ± 0,28
1,0 – 2,0	26	17,3	1,02 – 1,95	1,36 ± 0,26
>2,1	12	8	2,36 – 9,40	3,99 ± 2,14
TOTAL	150	39,3	0,13 – 9,4	1,63 ± 1,57

N.A.: not detected

Aflatoxin has the ability to cross the placenta, enabling contamination during pregnancy (ALVITO, SILVA, 2022). After birth, exposure may continue through breast milk if the mother has been exposed to the toxin. In Brazil, low levels of contamination have been recorded in breast milk and infant formula (ISHIKAWA et al., 2017; BARROS et al., 2024). However, elevated levels of exposure have been reported in countries such as Egypt, Kenya, Morocco, Nigeria, and Tanzania (ALVITO, SILVA, 2022). After the breastfeeding period, solid foods become potential sources of aflatoxin contamination. Recent studies indicate that the presence of this toxin is quite common in children's products, such as cereals, milk, and dairy products (AÇAR, AKBULU, 2024).

Recent evidence indicates that infants and children are particularly vulnerable to aflatoxins due to their lower body weight, limited ability to detoxify harmful substances, restricted diet, immature metabolism and elimination processes, and their high growth and development rates (AÇAR, AKBULU, 2024).

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The relevance of addressing this problem transcends individual health, becoming a global food security and public health challenge. Addressing it requires coordinated interventions focused on prevention, early diagnosis, and mitigation of its impacts.

CONCLUSION

The anti-AFB1 monoclonal antibody (ACM) produced in this study proved to be suitable for the development of an ic-ELISA method aimed at detecting AFB1. The method proved to be sensitive, specific and precise for application in infant urine samples, in addition to enabling the use of reduced volumes of organic solvents, making it less harmful to health. Preliminary results indicate that the levels of AFB1 contamination in children in the region of Londrina, Paraná State, Brazil, are low.

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