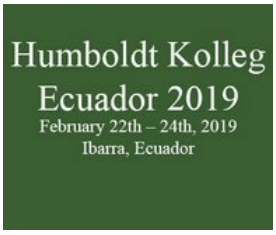
Bionatura ISSN: 1390-9355 Latin American Journal of Biotechnology and Life Sciences

Publications from the Humboldt Kolleg Ecuador 2019
"Breaking Paradigms: Towards a Multi-, Inter- and Transdisciplinary Science"
In commemoration of the 250th Anniversary of
February 21th – 24th, 2019.
Ibarra, Ecuador

250 Humboldt

Alexander von Humboldt
Stiftung/Foundation

CS 2019.02.01.6



Bionatura Conference Series Vol 2. No 1. 2019
"Breaking Paradigms: Towards a Multi-, Inter- and Transdisciplinary Science" In commemoration of the 250th Anniversary of Alexander von Humboldt

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Determination of Aflatoxin M1 concentration in volunteers from rural and urban areas of Cochabamba-Bolivia

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Available in: http://dx.doi.org/10.21931/RB/CS/2019.02.01.6

ABSTRACT

Aflatoxins are produced by toxigenic strains of *Aspergillus* fungi and include the subtypes: B1, B2, G1, and G2. Once ingested, aflatoxin is metabolized to aflatoxin M1 found in the urine. Because of the importance of aflatoxins on human health, we analyzed 550 urine samples collected from farmers and city volunteers from Cochabamba. The level of aflatoxin M1 was determined using the Helica Aflatoxin M1 ELISA Kit specific for urine. 216 volunteer farmers had an aflatoxin M1 range of 0.02-1.21 ng/ml, while 334 urine city volunteers had a range of 0.03-1.27 ng/ml, indicating a risk for exposure to aflatoxin contamination for both populations.

Keywords: Mycotoxins, Aflatoxins, *Aspergillus flavus, Aspergillus parasiticus*, ELISA

INTRODUCTION

Aflatoxins are harmful, secondary metabolites produced by toxigenic strains of fungi, including *Aspergillus flavus* and *Aspergillus parasiticus* [1-2]. These substances are considered highly carcinogenic, mutagenic, teratogenic and hepatotoxic for many living species including humans [3]. These strains have been found in different crops in the field, during harvest, transport, storage at home, or by occasional inhalation of these toxins through occupational exposure. Peanuts and corn are products that are easily and frequently contaminated. Aflatoxins are usually designated by letters, which refer to a physical characteristic, B1 and B2, and G1 and G2. Toxic strains of *Aspergillus flavus* typically produce only aflatoxin B1 and B2, whereas the toxigenic strains of *Aspergillus parasiticus* produce aflatoxins B1, B2, G1 and G2 [4]. Aflatoxin B1 (AFB1) has been classified by the International Agency for Research on Cancer (IARC) as a Group 1 carcinogen. Once AFB1 is ingested by humans, it is metabolized by liver enzymes into many metabolites, including aflatoxin M1 (AFM1) that can be found in urine samples. *Aspergillus flavus* and *Aspergillus parasiticus* are considered thermotolerant fungi. Although maximum levels and frequencies occur in

tropical and semi-tropical regions where the climate favors the growth of aflatoxin-producing fungi, contamination of food from temperate zones can occur as *Aspergillus flavus* is universally distributed and contamination of food with aflatoxins type B1 and B2 has been detected worldwide. From a health point of view, the finding of aflatoxins in food should be a public concern due to the carcinogenic power of aflatoxins predisposing individuals to the development of liver cancer, and long-term exposure may have adverse health effects. Globally, it is the seventh most frequently occurring malignant neoplasm for men and women combined in 2018 [5]. There is no innocuous level known for humans. The US Food and Drug Administration (FDA) standard for primary agricultural foods and their derivatives is 20 µg/kg of total aflatoxins. Studies carried out by the Valles Foundation Cochabamba-Bolivia indicate that the incidence of aflatoxins in peanuts has been reduced from 30% to 3% in the period from 2012 to 2014, through the application of the Prevention and Control System Aflatoxins (SIPCA) [6]. The families of the producers consume the peanut not suitable for commercialization; it is the discarded portion after the selection has been made. It is estimated that the level of aflatoxins in this discarded peanut is between 4 and 340 ppb. Bolivian Norm NB 32004 establishes <10 ppb of aflatoxin as maximum limit in peanuts and nuts for human consumption[12]. In Bolivia, there are no methods to monitor the exposure of aflatoxins and their metabolites in human urine samples, allowing us to establish risk criteria in the health status of people. The determination of AFM1 in urine will allow us to determine the amount of AFB1 ingested, being a good biomarker of recent exposure [2, 7-8]. In previous studies, our group has detected the presence of AFM1 in urine samples from 154 peanut producers at concentrations of 0.002 to 1.105 ng/ml and an average of 0.384 ng/ml. This study reveals the presence of aflatoxins in urine samples because these foods constitute a considerable part of the diet of consumers [6]. Our main objective in this research is to carry out a larger sampling in rural areas (volunteer farmers) and urban populations (city volunteers) in order to determine the group with the highest risk of exposure to contamination with aflatoxins. This baseline information is critical for developing strategies to mitigate aflatoxin consumption in populations that are at the highest risk.

MATERIALS AND METHODS

Ethics committee

This project has been approved by the research ethics committee of Hospital Albina R. de Patiño in the city of Cochabamba.

Project explanation seminars

In order to obtain the urine samples from volunteer farmers, the seminars explaining the Project have been carried out by the technical staff of the Valles Foundation and volunteers from the city of Cochabamba. The head of the Chagas disease and Immunoparasitology Laboratory and her technical group have initially made visits to different social assistance institutions in the city of Cochabamba, explained the purpose of the project in each institution in order to obtain permission to enter and analyze urine from each of the volunteer participants. The term of informed consent of all the participants and of their parents or guardians in the case of the minors has been signed.

Collection of urine samples

Urine samples were collected in the morning on an empty stomach, with prior instruction to each participant. The samples were filtered on Whatman No 3 filter paper in order to obtain the sample free of debris and precipitate.

Population of study

A total of 550 urine samples were collected, 216 of which were volunteer participants from the municipalities covered by the project “Exposure to aflatoxins in the diet of peasant families in the municipalities of Anzaldo, Aiquile and Mizque (Cochabamba); Icla, Villa Serrano, Alcalá, Padilla, El Villar and Tomina (Chuquisaca) and Torotoro (Potosí)” and 334 urine samples from volunteers from the city of Cochabamba.

Quantification of AFM1 in urine samples:

The level of aflatoxins has been determined using the commercial kit Helica Aflatoxins M1 ELISA (Quantitative assay for Aflatoxin M1 in urine, Helica Biosystems Inc., Santa Ana, CA) specific for urine samples. The procedure

of the ELISA method has been performed following the manual provided in the kit. Absorbance was measured at 450 nm using a microplate reader (Tecan, Reader, Sunrise, Austria). Standards and samples have been analyzed through a dose-response analysis for a toxic substance that can be plotted through a curve where the point at which toxicity appears is the “threshold dose level”. The shape and slope of the dose-response curve are extremely important in predicting the toxicity of a substance at specific dose levels. The dose-response relationship has allowed us to describe the distribution of responses at different doses in a population of individuals.

Enzyme-Linked Immunosorbent Assay (ELISA) for the detection of Aflatoxins M1 in urine

Collected samples have been previously filtered on Whatman No 3 filter paper. Samples and standards have been analyzed in duplicate. The polystyrene wells are precoated with antibodies with high affinity for aflatoxin M1. After initial dilution with distilled water, the urine sample is mixed with assay buffer and added to the well. If aflatoxin M1 is present in the urine, it will bind to the coated antibody. Subsequently, the peroxidase-bound aflatoxin is added which binds to the antibody sites not already occupied by the aflatoxin present in the sample or standard. After the incubation period, the contents of the wells are decanted, washed, an HRP substrate is added, and a blue color develops in the presence of the enzyme. The color intensity is directly proportional to the amount of conjugate bound and inversely proportional to the number of aflatoxins in the standard or sample. Therefore, when the concentration of aflatoxin in the sample or standard increases, the intensity of the blue color will be reduced. An acid stop solution is added which changes the chromogen color from blue to yellow. The microwells are measured optically by a microplate reader with an absorbance filter of 450 nm wavelength. The optical densities of the samples are compared with those of the standards of the kit and an OD result is determined by interpolation of the standard curve or expressed dose-response curve in ng/ml.

Analysis of the data

All the data has been placed in an EXCEL database for later analysis. A dose-response curve was constructed using the software, SOLVER CONVERT, with the aflatoxin standard optical density (OD) values containing the following concentrations of 0.0, 0.15, 0.40, 0.80, 1.50, and 4.00 ng/ml. When the standard curve or dose-response curve (Figure # 1) was constructed, the data from the samples were placed on the template created for later interpretation in ng/ml.

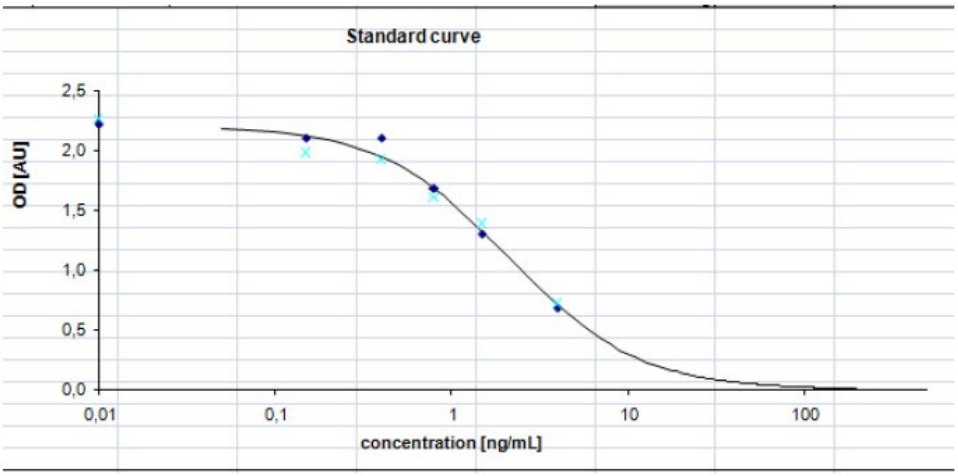

Figure 1: Dose-response curve

RESULTS AND DISCUSSION

Five hundred and fifty samples of urine were collected from fasting individuals (Table # 1). 216 correspond to samples that were analyzed from farmers in different municipalities of Cochabamba, Sucre, and Potosi, with aflatoxin range of 0.02 to 1.21 ng/ml; 334 urine samples correspond to volunteers from the city of Cochabamba, whose aflatoxin M1 ranges from 0.03 to 1.27 ng/ml. Of the 216 farmer samples, 109 samples were female with aflatoxin M1 concentrations ranging from 0.02 to 1.21 ng/ml, and 107 male samples with 0.07 to 1.04 ng/ml of aflatoxin M1. Of the 334 volunteer urine samples from the city of Cochabamba, 176 were female with 0.53 to 0.77 ng/ml, and 158 were male with 0.03 to 1.27 ng/ml of aflatoxin M1. The concentrations of aflatoxin M1 in ng/ml found in the present study group between rural volunteers (voluntary farmers) and urban volunteers (city volunteers) are very similar, although rural female volunteers have a lower concentration compared to the other groups. Our aflatoxin M1 concentration data in urine were comparable with those obtained in Malaysia. The levels they obtained were 0.0289 to 0.1547 ng/ml with mean value of 0.0421ng/ml (geometric mean = 0.0381 ng/ml) [9]. Since human

exposure to aflatoxin is related through diet, one study has reported a good correlation between the presence of AFB1 in the diet and the presence of AFM1 in urine samples [10]. Jolly *et al* [11] indicated that aflatoxin absorption from the diet is the crucial determinant of urinary levels of AFM1.

	FIELD			CITY		
	TOTAL	FEMALE	MALE	TOTAL	FEMALE	MALE
No. of samples	216	109	107	334	176	158
Aflatoxin M1 (ng/mL)	0.02-1.21	0.02-1.21	0.07-1.04	0.03- >1.27	0.53-0.77	0.03- >1.27
Mean (ng/mL)	0.43390314	0.41865208	0.44943925	0.48138578	0.53274948	0.42417053

	FEMALE	MALE	No. Of Samples	Aflatoxin sng/mL	FEMALE ng/mL	MALE ng/mL	MEAN total (ng/mL)	MEAN FEMALE (ng/mL)	MEAN MALE (ng/mL)
FIELD	109	107	216	0.02-1.21	0.02-1.21	0.07-1.04	0.43390314	0.41865208	0.44943925
CITY	176	158	334	0.03->1.27	0.53-0.77	0.03->1.27	0.48138578	0.53274948	0.42417053
TOTAL			550						

Table 1. Results of the analysis

CONCLUSION

The average concentration of aflatoxins in city volunteers is higher than that of volunteers in the field. The female population in rural areas has more aflatoxins than men, whereas in the city, the male population has more aflatoxins than female. The low values of aflatoxins obtained in the present study allow us to suggest that a constant control should be carried out even if the dose of aflatoxins ingested is small, knowing that contamination depends on many factors, such as lifestyle, intake by occupational exposure and environmental factors, such as temperature and humidity, suitable for the growth of Aspergillus sp. Genetic susceptibility and the nutritional status of individuals also play a role. Jolly et al. in 2006, 11 stated that the absorption of aflatoxins through the diet should be the crucial determinant of urinary levels of AFM1

RECOMMENDATIONS

The present research project has provided us with initial data to establish a study of aflatoxin biomarkers. The AFM1 aflatoxins analysis in urine samples should be recommended in health institutions as routine analysis, recommending that the finding should be a public concern because aflatoxin is a potent carcinogen and long term exposure may have deleterious effects for health since there is no known harmless level for humans. The initial results of this research work are an opening for education to the general population about the harmful effects of aflatoxins and their serious consequences.

ACKNOWLEDGMENTS

This research work has been supported by the Valles Foundations Cochabamba Bolivia under the project entitled "Exposure to aflatoxins in the diet of peasant families in the municipalities of Anzaldo, Aiquile and Mizque (Cochabamba); Icla, Villa Serrano, Alcalá, Padilla, El Villar and Tomina (Sucre) and Torotoro (Potosí) ", as part of the project to promote the production of healthy peanuts and maize. The authors have stated that they have no potential conflicts of interest with the exception of Thu Huynh, who works at Helica Biosystems, which manufactures the aflatoxin M1 in urine kit used for this study

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Received: 3 March 2019
Accepted: 28 May 2019

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