

Research Proposal: Mycotoxin Detection in Dried Blood

Description

Development and Validation of Methods for Detection of Mycotoxins Exposure in Dried Spotted Blood Samples

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Partner Scientists

Study samples will be provided by the following collaborative scientists:

Nii-Ayi Ankrah

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Justice Kumi

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Laurena Arone

Edwardo Mondlane University

Mozambique

Bob Kemerait and Tim Brenneman, Med & Food for Kids (MFK), Partners in Health, Haiti via Dan Brown at Cornell University, USA

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Tufts University, USA

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Geographical Locations

Method development and initial validation will be conducted in University of Georgia, USA, and validation phases will be conducted in samples collected from collaborators from Ghana, Haiti, Malawi, Mozambique, Zambia, and NILs in Africa and Asia.

Project Duration

September 1, 2013 to August 31, 2016

Executive Summary

The goal of this project is to establish and validate methods for measuring major mycotoxin biomarkers, especially for aflatoxin-lysine adduct, in human dried blood spot samples for supporting urgent needs of nutritional and intervention studies conducted in Asia and Africa countries by Peanut and Mycotoxin Innovation Laboratory as well as USAID-Nutritional Innovation Laboratories. Mycotoxins are toxic fungal metabolites produced mainly by toxicogenic fungi in the genera *Aspergillus*, *Penicillium*, and *Fusarium*. Several mycotoxins, such as aflatoxins and fumonisins, are ubiquitous food contaminants, especially in peanuts, other groundnuts, and corn. Human populations in developing world are frequently exposed to these toxicants through their regular diets. These important mycotoxins have been strongly associated with acute human toxicoses, growth retardation and developmental inhibition in children, immune suppressive effects, as well as increased rates of many types of primary cancers. Aflatoxins (AFs) are among the most potent and commonly occurring mycotoxins. Aflatoxin 81 (AFB1) is acutely toxic to all species of animals and humans, as evidenced by a recent outbreak in Kenya. Chronic aflatoxicosis is characterized by liver function damages, growth retardation and developmental inhibition in children, formations of liver cirrhosis and hepatocellular carcinoma (HCC). AFB1 has been classified as a

known human carcinogen by the International Agency for Research on Cancer (IARC). AFB1 also has anti-nutritional effect and reduced vitamins and proteins in animals and humans. Further, AFB1 has potent immunotoxic effect, which may aggravate the infectious diseases rate in developing world. Therefore, accurate assessment of AFs exposure and evaluation of the efficacy of various intervention strategies, including the outcome of peanut value chain changes, are critical for improving food safety and human health. In this proposed research project, we will develop and validate a sensitive and reliable method to analyze mycotoxin biomarkers, especially for AFB1-lysine adduct in human dried blood spots (DBS) samples to assess susceptibility factors in determination of human aflatoxicoses, to evaluate the linkage between AF exposure and human nutrition deficiency, growth retardation and developmental inhibition in children, and to examine roles of AF exposure in affect human immunity. DBS sampling technique was first developed to screen newborn babies for the genetic metabolic disorder phenylketonuria. This technique has more recently been applied to pharmacokinetic, therapeutic drug monitoring, and toxicokinetic studies, in an effort to reduce pharmaceutical expenses during drug development. The DBS sampling technique has several distinct advantages over conventional blood or plasma sampling in that it is less invasive, uses smaller blood volumes, utilizes simple storage methods, minimizes shipping expenses, offers convenient sampling, and the reduces risk of blood borne pathogens such as HIV. There is a significant push in recent years for use of DBS sampling technique in the field of biomedical research, including clinical and epidemiological studies. Thousands of human DBS samples have been collected in previous USAID supported studies and there is an urgent need to establish a highly sensitive and reliable method to evaluate mycotoxins exposures in use of these DBS samples. However, up to now there is no report about measurement of mycotoxins and their biomarkers in DBS due to many technical challenges. Therefore, this project is highly innovative and significant. Three phases or stages with clearly stated objectives are well defined in this proposal. Milestones and time line for each stage are also well described.

Project Description

Goal

The goal of this project is to establish and validate methods for measuring major mycotoxin biomarkers, especially for aflatoxin-lysine adduct, in human DBS samples for supporting urgent needs of nutritional and intervention studies conducted in Asia and Africa countries by Peanut and Mycotoxin Innovation

Laboratory as well as USAID-Nutritional Innovation Laboratories.

Relevance and Justification

Mycotoxins are toxic fungal metabolites produced mainly by toxicogenic fungi in the genera *Aspergillus*, *Penicillium*, and *Fusarium* (1). They are structurally diverse compounds and to date more than 350 parent toxins and their derivatives have been identified worldwide (2-4). Certain mycotoxins, such as aflatoxins, fumonisins, some tricothecenes, and ochratoxins have been implicated in highly lethal episodic outbreaks of mycotoxicoses in exposed animals and/or human populations (1-4). Aflatoxins (AFs) and fumonisins are ubiquitous food contaminants, especially in groundnuts and corn. Human populations in developing world are frequently exposed to these toxicants through their regular diets (1-5). They have been strongly associated with acute human toxicoses, growth retardation and developmental inhibition in children, immune suppressive effects, as well as increased rates of many types of primary cancers (1-5). AFs, produced by *Aspergillus flavus* and *A. parasiticus*, are among the most potent and commonly occurring food-borne toxicants. AFB1 is acutely toxic to all species of animals tested with LD₅₀ range of 0.3-9.0 mg/kg (1). Acute toxic effects in humans, as evidenced by a recent outbreak in Kenya, include vomiting, convulsions, coma, and death with cerebral edema and fatty accumulation in the liver, kidney, and heart (6, 7). Chronic aflatoxicosis is characterized by bile duct proliferation, periportal fibrosis, icterus and cirrhosis of the liver. Prolonged exposure to low levels of AFB1 leads to hepatocellular carcinoma (HCC) and other tumors in animals (1-3). The other significant adverse health effects are growth retardation and developmental inhibition in children (8-10). AFB1 is one of the most potent genotoxic agents in many model systems, including chromosomal aberrations, micronuclei, sister chromatid exchange, unscheduled DNA synthesis, chromosomal strand breaks, and formation of DNA and protein adducts (5). The predominant AFB1-DNA adduct is 8,9-dihydro-8-(N⁷-guanyl)-9-hydroxy-AFB1 (AFB-N⁷-gua), which derives from covalent bond formation between C8 of AFB1-8,9-epoxides and N⁷ of guanine bases in DNA (11). This initial AFB-N⁷-gua adduct can convert to a ring-opened formamidopyrimidine derivative, AFB-FAPyr (12). The major AFB1 protein adduct is AFB1-Iysine adduct in serum albumin (13). In addition, activation of the *ras* proto-oncogene has been found in AFB1-induced tumors in animal models (14, 15). Replication of DNA containing AFB-N⁷-gua adducts induced G \rightarrow T mutations in an experimental model (16). More strikingly, the relationship between AF exposure and development of human HCC

was demonstrated by studies on the *p53* tumor suppressor gene (17). High frequency of *p53* mutations (G7T transversion at codon 249) were found to occur in HCC tissues collected from populations with high dietary AF exposure in China and Southern Africa (18, 19). Furthermore, the role of synergistic interactions between AF and hepatitis B virus (HBV) in the formation of HCC has also been confirmed (20, 21). AFB1 has been classified as a known human carcinogen by the IARC (2, 3). AFB1 also has anti-nutritional effect and reduced vitamins and proteins in animals and humans (22-24). Further, AFB1 has potent immunotoxic effect, which may aggravate the infectious diseases rate in developing world (25-27).

While contamination by the AF-producing molds may be universal within a given geographical area, the levels or final concentration of AFs in the grain product can vary from <1 tJg/kg (1 ppb) to > 12,000 tJg/kg (12ppm) (5). For this reason, the measurement of human consumption of AF by sampling foodstuffs is extremely imprecise. Further, obvious contamination of a commodity with the fungi does not necessarily demonstrate the presence of AFs, and the appearance of a sound, uninfected sample of commodity does not preclude the existence of significant quantities of AFs (28, 29). Therefore, accurate assessment of AF exposure using the biomarker technique is urgently needed and highly justified. Over past 25 years, we have focused on development and validation of molecular biomarkers for aflatoxin exposure and adverse health effects (30-32). Serum AFB1-albumin adduct has been proven to be the most reliable molecular biomarker (33-35)). As a result of its longer in vivo half-life as compared to other urinary AFB metabolites, such as AFM1 and AFB-N7-Gua, AFB-albumin adduct can reflect integrated exposures over longer time periods. The long-term stability (up to 20 years) in frozen serum or plasma made this adduct a first choice of biomarkers to study linkage between AF exposure and disease (36-37). From a global view, the measurement of serum AFB-albumin adducts offers a rapid, facile approach that can be used to screen very large numbers of people. Highly significant associations between AFB-albumin adduct level and dietary AFB1 exposure has been found in human populations from several regions of the world. In addition, AFB-albumin adduct has been used as a biological response indicator of acute and chronic human diseases, such as aflatoxicosis in Africa, risk of HCC in Taiwan, China, and Africa, and infectious disease linked immune suppression (38-41). Moreover, AFB-albumin adduct has been used as the primary biomarker to assess efficacy of several human chemoprevention and intervention trials (42-44). Therefore, application of AFB-albumin adducts as optimal biological response

indicators for evaluation of global concern on human aflatoxicosis; especially in developing world is highly significant.

The DBS sampling technique was first developed to screen newborn babies for the genetic metabolic disorder phenylketonuria (45). This technique has more recently been applied to pharmacokinetic, therapeutic drug monitoring, and toxicokinetic studies, in an effort to reduce pharmaceutical expenses during drug development (46). The DBS sampling technique has several distinct advantages over conventional blood or plasma sampling in that it is less invasive, uses smaller blood volumes, utilizes simple storage methods, minimizes shipping expenses, offers convenient sampling, and the reduces risk of blood borne pathogens such as HIV (46-47). There is a significant push in recent years for use of DBS sampling technique in the field of biomedical research, including clinical studies (48-49). Up to now there is no report about measurement of mycotoxins and their biomarkers in DBS due to many technical challenges. There are thousands of DBS collected in previous USAID supported studies and it is urgently need to establish a reliable method to evaluate mycotoxins exposures in these DBS samples. Therefore, this project will focus on developing and validating methods for measuring major mycotoxin biomarkers, especially for aflatoxin-lysine adduct, in DBS samples. We will analyze human blood samples via DBS technique to assess susceptibility factors in determination of human aflatoxicoses, to evaluate the linkage between AF exposure and human nutrition deficiency, growth retardation and developmental inhibition in children, and to examine roles of AF exposure in affect human immunity using the validated method.

Research Plan

Objectives

Phase 1

Method Development Stage in Year 1

1. To compare capacity of DBS cards from different commercial sources for holding the whole blood, serum/plasma, and to optimize the washing strategy for recovery of all materials in DBS cards.
2. To establish methods to measure concentrations of total proteins and albumin in diluted micro-volume washing solutions and to optimize conditions of enzyme digestion to release aflatoxin-bound lysine adduct

from the protein.

3. To develop method for concentration and purification of aflatoxin-lysine adduct in digests for instrument analysis and to determine analytical chemistry parameters, such as accuracy, precision, sensitivity (limit of detection), reproducibility, and recovery for the method.

Phase 2

Validation Stage in Year 2-3

1. To validate the method in young adult animals (Fischer 344 rats) treated with a single dose (25, 50, 100, and 200 IJg/kg body weight) or repeated doses (5, 10, and 20 IJg/kg body weight for 5 weeks) of AFB1;
2. To validate the method in human blood samples collected in focused home countries, such as Ghana, Haiti, Malawi, Mozambique, and Zambia or in human DBS samples collected from on-going USAID-supported human intervention studies conducted by Nutrition Innovation Laboratories.

Phase 3

Application & Training Stage in Year 3 and beyond

1. To assess susceptibility factors in determination of human aflatoxicoses, to evaluate the linkage between AF exposure and human nutrition deficiency, growth retardation and developmental inhibition in children, and to examine roles of AF exposure in affect human immunity using the validated method.
2. To train scientists and technical people from focused countries and collaborative people from other Innovative laboratories.

Role of Each Scientist/Partner

Dr. Jia-Sheng Wang

Is a professor of public health and toxicology, and the head for the Department of Environmental Health Science, College of Public Health, University of Georgia. Dr. Wang received his M.D. in preventive medicine from the Shanghai First Medical College, China with the major in food-borne diseases. He received Ph.D. in Pathology and Immunology from the Boston University School of Medicine and did his postdoctoral training as a NIEHS fellow in molecular epidemiology at the Johns Hopkins University School of Public Health. For more than 30 years, he has focused on studying human health effect of exposure to mycotoxins, especially for AFs. He worked at Dr. G. N. Wogan's research group in MIT from 1986-1992 to develop a monoclonal antibody based immunoaffinity method for detecting AFs and other mycotoxins in food samples. He was the key research

scientist at J. D. Groopman's research group in the Johns Hopkins University to develop and apply AFB1 monoclonal antibody based immunoaffinity-HPLC-fluorescent and immunoaffinity-LC-MS methods for AF-related human epidemiological studies in China, Mexico, and Gambia, and chemoprevention studies in China. Mouse monoclonal antibody he developed against a synthetic AFB1-Iysine-cationized bovine serum albumin conjugate is one of the most sensitive antibodies for AF and associated biomarkers. Over past 10 years, under the support by the USAID (LAG-G-00-96-90013-00; ECG-A-00-0700001-00) grants through Peanut CRSP of the University of Georgia, Dr. Wang works closely with many investigators in US Institutions and home country institutions in Burkina Faso, Ghana, Haiti, and Uganda and has published over 30 peer-reviewed articles. He has been invited to present research findings in several international mycotoxin meetings including Mycoglobe and MYCORED meetings and IUPAC meeting. He also serves as guest professors for many international institutions and participate training courses organized by WHO, FAO, and IARC in different countries. Therefore, in collaboration with PMIL investigators and home country scientists, Dr. Wang has full capacity to be the principal investigator of the project for completion of the proposed method development, validation, and application phases. He will be responsible for the overall study planning, design, and implement of each phase of planned studies. He will also be responsible for the overall conduct and report of the progress to PMIL or USAID.

Dr. Lili Tang

Is an associate research scientist at the Department of Environmental Health Science, College of Public Health, University of Georgia. She received her BM in preventive medicine from the Southeast University, MS in food science from the Jiangnan University, and PhD in toxicology from Fudan University. She was a postdoctoral associate and an assistant research professor at Texas Tech University, and a professor in food safety at the Jiangnan University. She has been working with Dr. Wang for 11 years at the USAID/Peanut CRSP funded research projects. She, as the co-project investigator, will supervise and conduct daily work proposed for method development, analyses of biomarkers, and validation. She is also responsible for database establishment and statistical analysis.

Other Scientists

From collaborative institutions and focused countries will provide human DBS samples and will be engaged in the assessment of aflatoxin exposure and

adverse health effects in Phase 3.

Annual Work Plan, Milestones and Time Line

Year 01

We will focus on three objectives at the Phase 1 stage. We will use whole human blood, serum, and plasma to spot in different DBS cards obtained from different vendor sources, and test various washing conditions, including different buffers and solvents in different pH, temperature, and time in recovery of all blood components, especially for albumin and total proteins. Since the concentration of proteins is extremely low, we have to enhance the sensitivity for traditionally clinical measurement for albumin and total protein concentration, which is critical for final normalization. The ratio of enzyme and protein concentration will also be re-optimized. Instrument analysis is the critical for the method development and determination of various analytical parameters is essential for the reliability and applications of the method.

Tech approaches in Phase 1 method development will include step-wise procedures for release of mycotoxins, especially for AFB1-Lysine adduct from DBS samples via optimized washing procedure and enzyme digestion; solid-phase enrichment of the adduct using chemically selected polymer-based cartridge; HPLC separation, and followed by fluorescence detection, then LC/MS confirmation. Previously established methods (50-54) with human serum shows a recovery of higher 90% and the limit of detection at 0.1 pg/mL serum. The method for DBS will be kept in the similar sensitivity and other analytical parameters.

Milestones

1. Select appropriate DBS cards for blood collection
2. Develop automated washing conditions to recover mycotoxins and absorbed biomarkers
3. Establish methods to measure concentrations of total proteins and albumin in diluted micro-volume washing solutions
4. Optimize conditions of enzyme digestion to release aflatoxin-bound lysine adduct from the protein
5. Develop method for concentration and purification of aflatoxin-lysine adduct in digests for instrument analysis
6. Determine analytical chemistry parameters, such as accuracy, precision, sensitivity
7. (limit of detection), reproducibility, and recovery for the method

8. Publish one peer-reviewed article about development of the method

Year02

We will focus on two objectives at the Phase 2 stage. We will use the single-dose and repeated-dose study protocol to validate the relationship between exposure and biomarker levels via DBS sampling technique, which is very important to establish good dose-response relationship in exposure, sampling, and detection techniques. Human blood samples collected from our focused home countries, such as. Ghana, Haiti, Malawi, Mozambique, and Zambia via DBS sampling technique or in human DBS samples collected from on-going USAID-supported human intervention studies conducted by Nutrition Innovation Laboratories will be validated to test if the developed method meets the sensitivity and accuracy for human study applications.

Milestones

1. Complete single-dose animal study using young Fischer 344 rats treated with a single dose of aflatoxin 81 (25, 50, 100, and 200 $\mu\text{g/kg}$ body weight) to validate DBS sampling technique and validate analytical method for measurement of aflatoxin-lysine adduct (to examine dose-response relationship).
2. Complete the repeated doses animal studies in young Fischer 344 rats treated with repeated dose of AFB1 (5, 10, and 20 $\mu\text{g/kg}$ body weight for 5 weeks) to validate DBS sampling technique and validate analytical method for measurement of aflatoxin-lysine adduct (to examine temporal and accumulated dose-response relationship).
3. Complete a small sets of human blood samples collected from our focused home countries, such as Ghana, Haiti, Malawi, Mozambique, and Zambia via DBS sampling technique to validate the developed method (to examine the sensitivity and accuracy for human study applications).
4. Publish at least two peer-reviewed articles for validation of the method in animal and small set of human studies.

Year 03 and beyond

We will focus on two objectives at the Phase 3 stage, i.e. focus on application and training. We will apply the newly developed method to support various USAID-supported human studies for evaluation of linkage between AF exposure

and adverse health effects in pregnant women and children and will evaluate the efficacy of various intervention strategies in human populations, including the outcome of peanut value chain changes. We will analyze human blood samples via DBS technique to assess susceptibility factors in determination of human aflatoxicoses, to evaluate the linkage between AF exposure and human nutrition deficiency, growth retardation and developmental inhibition in children, and to examine roles of AF exposure in affect human immunity using the validated method. Further, we will train focused countries' scientists and collaborative institutions' technical people via short courses. These courses will use standard materials, standard operating procedures, and good laboratory practice guidelines to train participating people for use of the analytical method and for the overall study design on a longitudinal human biomonitoring study for aflatoxin exposure, including Institutional Review Board Approval, sample collection, separation, storage, transportation, analysis, and final data entry and report.

Milestones

1. Complete analysis of at least 2500 DBS samples for assessment of susceptibility factors in determination of human aflatoxicoses, for evaluation of the linkage between AF exposure and human nutrition deficiency, growth retardation and developmental inhibition in children, and for examination of roles of AF exposure in affect human immunity using the validated method.
2. Train 5-8 scientists and technical people from focused countries and collaborative Innovative laboratories.
3. Publish at least three peer-reviewed articles for application of the method in different human populations with different specific aims.

Project Evaluation Plan

The project will be evaluated in each of proposed phases with specific focus on evaluation of deliverables (milestones). The project will provide annual progress report for administrative evaluation. Quality will be assessed in the presentation prepared for the annual principal investigators' meeting. Evaluation will also be made to exam compliance and publications.

Gender Research Strategy

Gender is a very important determinant, not only in economic development and human right, but also in agricultural and biomedical research. Variations of

gender in biochemical process, physiological characteristics, and pharmacological responses to nutrients and chemicals have been documented for many years. Human exposure to environmental toxicants, including mycotoxins, is strongly influenced by gender. Hormonal regulation in absorption, distribution, and biotransformation (metabolism) as well as biological effects is a significant determinant to modulate every step of human development, growth, and pathogenesis of diseases. Consideration of gender issue should be included in all research projects.

Gender issue has been considered in the proposed research project. In the phase 1 of the project, blood samples pooled from both females and males will be used for development of method; in the phase 2 of the project, validation studies in animals will use both male and female animals; gender balance will be considered to conduct a small set of human DBS samples; in the phase 3 stage, gender factor will be considered in the application studies and intervention studies (recruitment of participants with both gender). Gender will be considered as an independent factor for data analysis and final report.

Outcomes and Impacts

Mycotoxins, especially aflatoxins, are ubiquitous food-borne toxicants, and human populations in many parts of the developing world are frequently exposed to these chemicals through contaminated food. These toxicants have been strongly associated with human aflatoxicosis as well as many types of human diseases, including primary cancers. The consequences of AF exposure as a factor (solely or in combination with hepatic viruses) increasing the risks of HCC are well documented (IARC, 1993; 2002), but until recently the nutritional and immunological consequences have been largely neglected. AF contamination in food products remains a serious burden in developing countries where poverty and contaminated food supplies present a major and persistent public health challenge. Therefore, accurate assessment of AF exposure and its linkage to chronic adverse health effects in human populations, especially in highly vulnerable populations such as pregnant women and their children is urgently needed and highly significant. In this research proposal, we propose to develop and validate method to measure mycotoxins biomarkers, especially for aflatoxin-lysine adduct, in human dried blood spots (DBS) samples in order to support ongoing USAID- Innovation Laboratories conducted studies in Africa, Asia, and Latin America developing countries for assessment of the potential adverse effect of exposure to dietary aflatoxin on various maternal and infant outcomes, including intrauterine growth retardation, maternal anemia, infant

and child growth, and other disease incidence. The DBS sampling technique has more recently been widely applied to biomedical and epidemiological research because the technique has several distinct advantages over conventional blood or plasma sampling: it is less invasive, uses smaller blood volumes, utilizes simple storage methods, minimizes shipping expenses, offers convenient sampling, and the reduces risk of blood borne pathogens such as HIV. Thousands of human DBS samples have been collected in previous USAID supported studies and there is an urgent need to establish a highly sensitive and reliable method to evaluate mycotoxins exposures in use of these DBS samples. However, up to now there is no report about measurement of mycotoxins and their biomarkers in DBS due to many technical challenges. Therefore, this project is highly innovative and significant. Upon completion, a sensitive and reliable method will be established, the method will be validated in both animal studies and small set human studies with both gender included. Application of this method will generate sufficient data for understanding the relationship between biological response indicators of mycotoxin exposure and genetics and non-genetic factors that contribute to human mycotoxicoses. This will have significant benefits for the risk assessment of human health effects as a result of long-term exposure to these mycotoxins. Data generated from our study will be a gold standard to evaluate adverse human health effects linked to environmental exposure to toxicants, which are highly significant for intervention and prevention strategies in the developing world. In addition, this project will train 5-8 scientists and technical people from the PMIL focused countries and other collaborative Innovative laboratories.

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