Development and Validation of Methods for Detection of Aflatoxin-Lysine Adduct in Dried Blood Spot Samples

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Aflatoxins

- A group of potent mycotoxins produced mainly by *Aspergillus flavus* and *A. parasiticus*;
- Widespread food contaminants, especially for corn & corn products, peanuts & other groundnuts, and rice;
- Human aflatoxicosis and hepatocellular carcinoma;
- Immunosuppressors;
- Anti-nutritional agents;
- Inhibition of children growth and development.
Metabolic Pathways of AFB₁

AFM₁ → CYP1A2 → AFB₁ → Phase I → AFB₁-8,9-epoxide → Phase II + H₂O → AFB₁-diol

AFB₁-diol → AFB₁-Lysine Adduct

AFB₁-Lysine Adduct → AFB₁-N⁷-Gua → AFQ₁ → CYP3A4
Exposure
Internal Dose
Biologically Effective Dose
Early Biological Effect
Altered Structure/Function
Chronic Disease

Interventions

Exposure biomarkers
Susceptibility markers
Effect biomarkers

Foodborne
Waterborne
Airborne
Original Goals

• To establish and validate methods for measuring major aflatoxin biomarkers in human dried blood spot (DBS) samples;

• To support needs of aflatoxin exposure assessment in USAID supported Peanut and Mycotoxin Innovation Laboratory (PMIL) and Nutrition Innovation Laboratory (NIL) research projects.
Working Hypothesis

• Levels of AFB$_1$-lysine adduct in human blood or DBS samples are correlated to dietary aflatoxin exposure and will be a reliable effective biological response indicator for aflatoxin-linked adverse health effects in high-risk human populations.
Background Information

• DBS sampling technique was first developed to screen newborn babies for the genetic metabolic disorder, phenylketonuria.

• DBS has been applied to nutritional supplement studies and pharmacokinetics’ studies during new drug development.

• DBS has been used for various “omics” studies.

• DBS has been proposed to use for HIV and HCV research and various environmental exposure studies.
Advantages of DBS Technique

- Less invasive;
- Uses smaller blood volumes;
- Utilizes simple storage methods;
- Minimizes shipping expenses;
- Offers convenient sampling;
- Reduces risk of blood-borne pathogens such as HIV, etc.
Regular methods

Dried Blood Spots

~ 3ml Blood

5 x 50 μl Blood

150μl serum aliquot
Challenges

- Hold enough mycotoxin or test target?
- Sensitivity?
- Specificity?
- Accuracy?
- Platform analysis for large quantity of samples?
- Acceptation?
Phase 1 Objectives

• To compare capacity of DBS cards from different commercial sources for holding the whole blood, serum/plasma, and to optimize the elution procedure for recovery of all materials in DBS cards.

• To establish methods to measure concentrations of total proteins and albumin in diluted micro-volume eluting solutions and to optimize conditions of enzyme digestion to release aflatoxin-bound lysine adduct from the albumin.
Phase 1 Objectives (continued)

• To develop method for concentration and purification of aflatoxin-lysine adduct in digests for instrument analysis;

• To determine analytical chemistry parameters, such as accuracy, precision, sensitivity (limit of detection), reproducibility, and recovery for the method.
Commercial DBS Card Comparison

**Untreated Cards**
- Ahlstrom 226
- Munktell TFN
- GE Whatman DMPK C - 31ETF base paper
- GE Whatman 903

**Treated Cards**
- GE Whatman DMPK A (FTA) – 31ETF base paper
  - 4 additives, for ‘protection’ of genetic material
- GE Whatman DMPK B (FTA Elute) – 903 base paper
  - 1 additive, will denature proteins
Main Supplies
Unsatisfactory Specimens

- Supersaturated
- Specimen Not Dried Before Mailing
- Serum Rings
- Clotted or Layered
- Quantity Insufficient for Testing
- Scratched or Abraded
- Diluted, Discolored, or Contaminated
- No Blood
Selection of DBS Extraction System

Manual
Lab Procedure

1. Cut out dried blood spot
2. One spot per well
3. 50ul sample per spot
4. Dry 3 hr and store
5. Shake plate on plate shaker, speed level 6, for 30 minutes.
6. Extract and store eluant in Eppendorf tubes.
7. Centrifuge 3000rpm 5 minutes
8. Process for HPLC analysis
9. Repeat PBS elution and combine eluants
Method Developed

- Sample collection: ≤ 50 µl blood in DBS card
- Washing Solution: Triton-100-PBS and rebuild the sample solution;
- Determination of albumin and total protein concentrations;
- Pronase digestion, solid-phase concentration and purification;
- HPLC-fluorescent detection, and MS confirmation;
- Limit of detection: 20 fg/mg albumin;
- Recovery: averaged 75% for various spiked concentrations.
# Elution & Washing Efficiency

## Total Protein (µg)

<table>
<thead>
<tr>
<th>Serum</th>
<th>Dilute to 1.5ml</th>
<th>Serum Spot Eluent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wash 1</td>
<td>Wash 2</td>
</tr>
<tr>
<td>20µl</td>
<td>1365.61</td>
<td>1409.63</td>
</tr>
<tr>
<td>40µl</td>
<td>2731.23</td>
<td>2644.29</td>
</tr>
<tr>
<td>60µl</td>
<td>4096.84</td>
<td>4131.43</td>
</tr>
</tbody>
</table>

Triplicate experiments
# Elution & Washing Efficiency

<table>
<thead>
<tr>
<th>Serum (ul)</th>
<th>Dilute to 1.5ml</th>
<th>Total Albumin (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum</td>
<td>Wash 1</td>
</tr>
<tr>
<td>20ul</td>
<td>884.13</td>
<td>649.17</td>
</tr>
<tr>
<td>40ul</td>
<td>1768.27</td>
<td>1606.67</td>
</tr>
<tr>
<td>60ul</td>
<td>2652.40</td>
<td>2575.83</td>
</tr>
</tbody>
</table>

Triplicate experiments
Phase 2A: Animal Validation Studies

Fisher 344 rats

Single-dose of AFB$_1$ (0, 25, 75 or 225 μg/kg BW)

Blood collection at 2, 4, 8 h and 1, 3, 5, 7, 14 and 21 days

Repeated doses of AFB$_1$ (0, 5, 10, 25 and 75 μg/kg BW)

Blood collection at 1, 2, 3, 4 and 5 weeks

Matched Serum and DBS Collected

HPLC Analysis
DBS samples from AFB₁-dosed animal blood

Whole blood DBS sample from high dose treated animals

Whole blood DBS sample from low dose treated animals
HPLC chromatograph of AFB$_1$-treated rat DBS

- Single dose of 225 µg/kg AFB1
- Single dose of 75 µg/kg AFB1
- Single dose of 25 µg/kg AFB1
## Single dose

<table>
<thead>
<tr>
<th></th>
<th>2h</th>
<th>24hr</th>
<th>3d</th>
<th>5d</th>
<th>7d</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.02±0.00</td>
<td>0.02±0.00</td>
<td>0.02±0.00</td>
<td>0.02±0.00</td>
<td>0.02±0.01</td>
</tr>
<tr>
<td>25μg/kg</td>
<td>16.48±2.58</td>
<td>5.62±0.42</td>
<td>5.90±1.02</td>
<td>2.83±0.16</td>
<td>1.34±0.16</td>
</tr>
<tr>
<td>75μg/kg</td>
<td>54.8±0.53</td>
<td>12.77±1.68</td>
<td>15.11±2.49</td>
<td>8.71±2.03</td>
<td>5.19±0.79</td>
</tr>
<tr>
<td>225μg/kg</td>
<td>143.98±20.45</td>
<td>96.19±10.67</td>
<td>80.72±5.80</td>
<td>66.63±16.91</td>
<td>36.18±7.57</td>
</tr>
</tbody>
</table>

N=6

## Repeated Dose

<table>
<thead>
<tr>
<th>Dose (μg/kg)</th>
<th>AFB-Lys (ng/mg alb.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>week 1</td>
</tr>
<tr>
<td>0</td>
<td>0.02±0.00</td>
</tr>
<tr>
<td>5</td>
<td>0.17±0.01</td>
</tr>
<tr>
<td>10</td>
<td>0.51±0.02</td>
</tr>
<tr>
<td>25</td>
<td>1.44±0.11</td>
</tr>
<tr>
<td>75</td>
<td>2.02±0.13</td>
</tr>
</tbody>
</table>

N=6
Single Dose

DBS ~ group + time

Superpose

time
2h
24h
3d
5d
7d
Scatter plots (left) and Bland–Altman plots (right) for paired serum and DBS specimens measured by HPLC. In scatter plots, solid line = linear regression. In Bland–Altman plots, center line indicates mean difference between serum and DBS measures; upper and lower lines indicate the 95% confidence interval.
Repeated Doses

DBS ~ Group + Time

Time
1-week
2-week
3-week
4-week
5-week

Group

0 20 40 60

DBS

0 0.5 1.0 1.5 2.0 2.5 3.0

superpose
Repeated Dose

Scatter plots (left) and Bland–Altman plots (right) for paired serum and DBS specimens measured by HPLC. In scatter plots, solid line = linear regression. In Bland–Altman plots, center line indicates mean difference between serum and DBS measures; upper and lower lines indicate the 95% confidence interval.
## Phase 2B. Human Validation Study

<table>
<thead>
<tr>
<th>Dietary aflatoxin exposure</th>
<th>Low</th>
<th>Middle</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participant numbers</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Detection rate (%)</td>
<td>50 (6/12)</td>
<td>58.3 (7/12)</td>
<td>100 (12/12)</td>
</tr>
<tr>
<td>Median level of AFB-lysine adduct (pg/mg albumin)</td>
<td>3.92</td>
<td>12.18</td>
<td>136.26</td>
</tr>
<tr>
<td>Range of AFB-lysine adduct (pg/mg albumin)</td>
<td>0-4.78</td>
<td>0-24.64</td>
<td>61.49-992.42</td>
</tr>
</tbody>
</table>
Human Validation Study

Scatter plots (left) and Bland–Altman plots (right) for paired human serum and DBS specimens. In scatter plots, solid line = linear regression. In Bland–Altman plots, center line indicates mean difference between serum and DBS measures; upper and lower lines indicate the 95% confidence interval.
Phase 3. Application Study

Use of DBS samples collected from USAID NIL conducted Aflatoxin Birth Cohort Study in Nepal to assess mother/children aflatoxin exposure and its correlation with adverse growth/development effects.
# AFB-Lys Adduct Levels in Five Batches of DBS Samples from Nepal Birth Cohort Study

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Batch 1</th>
<th>Batch 2</th>
<th>Batch 3</th>
<th>Batch 4</th>
<th>Batch 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB-Lys Adduct (pg/mg albumin)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>171</td>
<td>128</td>
<td>320</td>
<td>222</td>
<td>222</td>
</tr>
<tr>
<td>Detection rate (%)</td>
<td>98.83</td>
<td>96.88</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Median</td>
<td>1.65</td>
<td>1.21</td>
<td>2.99</td>
<td>8.64</td>
<td>15.68</td>
</tr>
<tr>
<td>Geometric Mean</td>
<td>4.24</td>
<td>1.77</td>
<td>2.92</td>
<td>8.94</td>
<td>14.71</td>
</tr>
<tr>
<td>95% CI</td>
<td>3.92 - 4.57</td>
<td>1.51 - 2.04</td>
<td>2.76 - 3.07</td>
<td>8.49 - 9.42</td>
<td>13.88 - 15.88</td>
</tr>
<tr>
<td>Minimal</td>
<td>0.40</td>
<td>0.20</td>
<td>0.43</td>
<td>3.51</td>
<td>2.92</td>
</tr>
<tr>
<td>Maximal</td>
<td>147.32</td>
<td>14.10</td>
<td>75.31</td>
<td>40.25</td>
<td>44.85</td>
</tr>
</tbody>
</table>
Distribution of AFB-Lys adduct Levels in DBS Samples of Nepal Birth Cohort

P<0.001
Outcomes and Impacts

- Highly innovative and significant;
- Meet urgent needs;
- Fill the research gaps;
- Generate data for understanding the relationship between biological response and aflatoxin exposure.
- Significantly beneficial for the health-effect assessment of children as a result of long-term exposure to aflatoxins in developing world.
Acknowledgement

- USAID Feed the Future Program Nutrition and Peanut/Mycotoxin Innovation Laboratories
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- Tufts University
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- Collaborators from NIL and PMIL Focused Countries
  - Nepal site
  - Uganda site