PROPOSAL NARRATIVE

Background: Contamination of food and animal feed with naturally-occurring fungal toxins (mycotoxins) is a major food safety concern worldwide. Although the exact figures for global losses from mycotoxin contamination may never be available, the Food and Agriculture Organization has estimated that mycotoxins affect 25% of the world's crops annually, resulting in the loss of approximately one billion metric tons of food and food products[1]. Dominant among fungal toxins are aflatoxins[2,3], which are produced by ubiquitous fungi within the genus *Aspergillus* that infest many agricultural commodities – especially the staple crops, peanuts and corn[2,3]. Aflatoxins are potently toxic to all animals, including humans. About 4.5 billion people in developing nations are chronically exposed to aflatoxins[4]. Depending upon the amount and frequency, ingestion is lethal, causes cancer[5], or exacerbates the symptoms of HIV[4,6] and hepatitis[4].

Climate change is altering the demographics of plant pathogens[7,8,9]. Pathogens, including *Aspergillus*, that were formerly relegated to lower latitudes are surviving and flourishing at higher latitudes. For example, the severely hot, dry growing season of 2012 resulted in an unusual and widespread aflatoxin contamination of US corn crops[10,11], resulting in large amounts of aflatoxin-contaminated corn that was unacceptable for food, feed, or biofuels, and therefore were dumped or diluted with toxin-free corn. Afterwards, despite these measures, aflatoxin was detected in pet food in the US and milk in Europe[12]. Testing to determine aflatoxin levels typically is done at harvest, when little can be done except to discard the crops. Pre-harvest testing is formally possible, and relies on a method called the polymerase chain reaction (PCR)[13,14]. Both post-harvest testing for aflatoxin and pre-harvest tests that involve PCR require skilled personnel working with expensive instruments in centralized laboratories, and thus such tests require time, infrastructure, and funds – all of which may be lacking in regions most affected by aflatoxins.

Vision and potential impact: I am collaborating with Scott Shibata and Drs. Ryo Kubota and Daniel Jenkins of Diagenetix, Inc., and Dr. Carla Garzón of the National Institute for Microbial Forensics & Food and Agricultural Biosecurity, to develop a low-cost, low-test, in-field method for predictive testing which could guide measures to prevent *Aspergillus* growth (and subsequent aflatoxin contamination) from increasing prior to harvest. We envision kits that could be useful both in the developed and developing world for aflatoxin risk assessment during the crop growing season. These kits would: 1) specifically detect and estimate the relative abundance of toxigenic *Aspergillus* genotypes, 2) be added to existing predictive tools (e.g. disease forecasting algorithms) for aflatoxin risk assessment, and 3) be used easily by growers for timely biocontrol and/or fungicide applications in precision agriculture.

Our method is based on a low-tech method called loop-mediated isothermal amplification of DNA (LAMP)[15]. Unskilled personnel can perform the test, because our method requires extraction of DNA rather than of toxic aflatoxin, and requires only simple instrumentation. The sensitivity of LAMP is comparable to quantitative PCR (Q-PCR), which currently is the gold standard for DNA-based pathogen detection. However, while Q-PCR requires an expensive thermocycler that changes reaction temperatures several times each minute, LAMP reactions are isothermal[15], and a simple device like a Thermos filled with a known volume of boiling water and an insert of a known mass can provide the correct incubation temperature[16]. In addition, LAMP and Q-PCR both target the region of DNA to be amplified via "primers" (small pieces of DNA complementary to a specific region in the genome). LAMP primers are more specific than conventional Q-PCR primers because they comprise a set of six, rather than two, sequences on the target DNA. Moreover, LAMP reactions are faster than Q-PCR, requiring only 15-30 minutes for completion due. Finally, LAMP results can be quantified via standard DNA detection methods, including via fluorescent DNA probes[17]. This latter option is exploited by Smart-DART technology, invented by Diagenetix, Inc.[18]. For Smart-DART detection of LAMP products, the LAMP reaction mixture must contain a fluorescent indicator that binds the DNA product. Fluorescence is detected by a hand-held reader that plugs into a smart phone. The portability and affordability of this system would enable distributed diagnostics across disparate geographies, rather than requiring that samples are collected and processed at a centralized laboratory. The speed of test results would enable a quicker response to mitigate the spread of the pathogen.

Objectives and current status of project: The crux of a successful LAMP protocol is the design of species- or strain-specific primers, and the optimization of a robust assay for each primer set. Herein, I propose to test a prototypical set of aflatoxin gene-specific LAMP primers for compatibility with Smart-DART technology. The work will be done with the assistance of undergraduate researchers at the junior or senior level, majoring in cell and molecular biology.

Obj. 1. Design LAMP primers to detect *Aspergillus* strains regardless of toxigenicity. Nontoxigenic strains of *Aspergillus* (with missing or mutated aflatoxin biosynthetic genes) coexist alongside toxic strains, especially now that the nontoxigenic *Aspergillus* strain Afla-Guard (NRRL 21882, lacking the entire aflatoxin gene cluster) is commercially available for biocontrol. *Thus, a robust test must be capable of differentiatinghigh-risk toxin-producing from non-producing strains.* Our lab identified several regions in the *Aspergillus* genome that might differentiate toxin-producing from non-toxic species and strains, and which are of a size useful for LAMP.

Current status and timeline: We confirmed that the 35S ribosomal RNA gene was optimal for *Aspergillus* detection. We designed eight primer sets for use in the LAMP reaction.

Work remaining: These 35S primers require testing and optimization for use with the Diagenetix reaction mixture. This will occur during the 2014-15 academic year. Should none of the eight primer sets work, I will need to consult with Dr. Garzón and design alternative primers during Summer 2015.

Obj. 2. Design primers that specifically detect aflatoxin-producing *Aspergillus* strains, by targeting the aflatoxin biosynthetic genes.

Current status and timeline: Toxic *Aspergillus* strains carry the aflatoxin biosynthetic genes. Our lab has designed seventeen LAMP primer sets to four genes in the aflatoxin biosynthetic pathway: *aflD*, *aflO*, *aflR*, and *aflP*. We have primer sets working reliably to *aflD* and *aflO*.

Work remaining: Optimization is necessary for binary positive/negative results with *aflR* and *aflP* and compatibility with the reaction mix provided Diagenetix. This work will require consultation with the inventor of the Diagenetix reaction mixture, Dr. Ryo Kubota, and will extend from the present time into Summer 2015.

Obj. 3. Confirm identity of amplification products: We will verify that the LAMP amplification products represent the correct target genes via either or both of two standard molecular methods: Southern hybridization and/or DNA sequencing analysis.

Current status and timeline: This work will take place during the summer of 2015.

Obj. 4. Query the sensitivity and specificity of our primer and probe sets. We will perform LAMP and Q-PCR simultaneously on serially diluted, pure and mixed template DNAs (representing varying ratios of aflatoxin producing and non-producing strains) to ensure comparable results and sensitivity between the LAMP method and the current gold standard for pathogen detection, Q-PCR.

Current status and timeline: This work will take place during the summer of 2015.

Enpoints and deliverables: This work is expected to yield a set of prototype LAMP primers that work with the Diagenetix Smart-DART platform. These primers should reliably detect 1) a region of the *Aspergillus* ribosomal RNA that occurs in both nontoxic and toxin-producing species and 2) the aflatoxin biosynthetic pathway genes *aflR*, *aflD*, *aflO*, and *aflP*. Success in Objectives 1-4 above would justify and guide the further development by Diagenetix, in collaboration with myself and Dr. Carla Garzón, of marketable aflatoxin detection kits for the Smart-DART platform for field use by growers. Further development would require external funding as described below.

Potential for Funding: This work has attracted funding (for July – December 2014) from the Washington Research Foundation, an organization that facilitates the collaboration of Washington university faculty and industry partners to bring products to the marketplace. My collaborators and I plan to apply for funding via small business technology transfer (STTR) grants from both the US Department of Agriculture (USDA) and the National Institutes of Health (NIH), within the Food and Drug Administration's Center for Food Safety and Applied Nutrition program.

REFERENCES

1. Schmale, D.G. III and G.P. Munkvold. 2014. Mycotoxins in Crops: A Threat to Human and Domestic Animal Health. http://www.apsnet.org/edcenter/intropp/topics/Mycotoxins/Pages/default.aspx
2. Kumar, V., M.S. Basu and T.P. Rajendran. 2008. Mycotoxin research and mycoflora in some commercially important agricultural commodities. Crop Prot. 27: 891-905.
3. World Health Organization. 2006. Mycotoxins in African foods: Implications to food safety and health. AFRO Food Safety Newsletter, July, 2006. http://www.afro.who.int/des.
4. Williams, J.H., T.D. Phillips, P.E. Jolly, J.K. Stiles, C.M. Jolly and D. Aggarwal. 2004. Human aflatoxicosis in developing countries: A review of toxicology, exposure, potential health consequences and interventions. Am. J. Clin. Nutr. 80: 1106-1122.
5. Groopmann, J.D. and T.W. Kensler. 1999. The light at the end of the tunnel for chemical-specific biomarkers: Daylight or headlight? Carcinogenesis 20: 1-11.
6. Gong, Y.Y., S. Egal, A. Hounsa, S. Egal, A. J. Hall, K.F. Cardwell and C.P. Wild. 2003. Determinants of aflatoxin exposure in young children from Benin and Togo, West Africa: The critical role of weaning. Int. J. Epidemiol. 32: 556-562.
7. Battisti DS, Naylor RL. 2009. Historical warnings of future food insecurity with unprecedented seasonal heat. Science 323: 240-244.
8. Coakley SM, Scherm H, Chakraborty S. 1999. Climate change and plant disease management. Ann Rev Phytopath 37: 399-426.
9. Paterson RRM, Lima N. 2010. How will climate change affect mycotoxins in food? Food Research International 43:1902-1914.
10. Bienkowski, B. 2012. 2012 drought: pick your poison. The Daily Climate, Oct. 1, 2012. Available at: http://www.dailyclimate.org/tdc-newsroom/2012/10/drought-toxins
11. http://otscweb.tamu.edu/Risk/Aflatoxin/Levels.aspx
12. http://en.wikipedia.org/wiki/2013_aflatoxin_contamination
13. Mayer, Z., Bagnara, A., Färber, P., and R. Geisen. 2003a. Quantification of the copy number of *nor-1*, a gene of the aflatoxin biosynthetic pathway, by real-time PCR, and its correlation to the cfu of *Aspergillus flavus* in foods. Int. J. Food Microbiol. 82:143-151.
14. Mayer, Z., Färber, P., and R. Geisen. 2003b. Monitoring the production of aflatoxin B_1 in wheat by measuring the concentration of *nor-1* mRNA. Appl. Env. Microbiol. 69:1154-1158.
15. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, and T. Hase. 2000. Loop-mediated isothermal amplification of DNA. Nucl. Acids Res. 28:e6311.
16. Kubota, R., LaBarre, P., Weigl, B.H., Li, Y., Haydock, P., and Jenkins, D.M. 2013. Molecular diagnostics in a teacup: Non-Instrumented Nucleic Acid Amplification (NINA) for rapid, low cost detection of *Salmonella enterica*. Biochemistry and Molecular Biology 58: 1162-1168.
17. http://loopamp.eiken.co.jp/e/lamp/index.html
18. http://diagenetix.com/