Rapid and sensitive detection of aflatoxins using immunomagnetic bead-based recovery and real-time qPCR assay

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STORY IN BRIEF

Several mold species of *Aspergillus* produce secondary metabolites called Aflatoxins. These aflatoxins can be carcinogenic and are considered potential threats to human and animal health when ingested. Aflatoxins occur naturally in food and feed products in microgram to milligram per gram of food or feed quantities thus detection methodology should be sensitive and specific which is not obtained with currently available methods. This study involves detection and quantification of aflatoxins in food products and animal feeds. Our research methodology involves use of immunomagnetic beads combined with real time PCR assay using aflatoxin specific polyclonal and monoclonal antibodies to capture and detect aflatoxins. Primary (polyclonal) antibodies for aflatoxin B1 were covalently attached to 2.8 μm-diameter magnetic beads using a bi-functional cross linking agent. A secondary antibody for aflatoxin B1 was also covalently linked to DNA oligonucleotides based on the *luciferase* gene as a reporter DNA molecule. Real-time PCR amplification of the reporter DNA after aflatoxin capture provides for sensitive detection of toxin, if present. Magnetic beads were coupled to primary antibodies while secondary antibodies were coupled to DNA reporter molecules. Amplification (PCR) targeting an internal portion of the reporter molecule gave DNA fragments of the expected size. Coating of magnetic beads with capture antibodies has been facilitated by use of the BeadRetrieverTM. Quantification of toxins in food and feed samples will involve signal amplification strategies using real-time PCR amplification of reporter DNA using specific primers. This methodology is rapid, sensitive, and will help to detect and quantify low levels of aflatoxins that could otherwise be fed to food production animals, domestic animals (cats, dogs, horses), or humans through aflatoxin-contaminated cereal grains and feed.

Key Words: aflatoxins, immunomagnetic bead capture, food safety, real-time PCR assay

INTRODUCTION

Microbial foodborne hazards include toxins that may have immediate (enterotoxins) or long term (aflatoxins) effects on the consumer. The toxins may be present in the processed food even after the producing organisms have been killed or removed. Aflatoxins are persistent mycotoxins produced when food or feeds are colonized by *Aspergillus flavus* or *A. parasiticus*. There are 13 known types of aflatoxins and type B1, B2, G1, G2 are the major ones encountered in most agronomic crops of cereals, oilseeds, spices, and tree nuts. Metabolites (types M1 and M2) of aflatoxins may appear in milk when the lactating animals are fed with aflatoxin contaminated feed (Lynch, 1972). Thus, aflatoxins are known to enter the food supply at any point of production, processing, transport and storage of food products or animal feed.

Due to the proven health hazards, the US Food and Drug Administration has set tolerance limits for aflatoxins in various commodities (0.5 ppb for milk, 20 ppb for all feedstuffs, 300 ppb for cottonseed meat (as feed ingredient), 100 ppb for breeding beef cattle, swine and poultry, 200 ppb for finishing swine, and 300 ppb for finishing beef (CAST, 2003)). Complying with the FDA regulations, the food and feed industries are in constant pursuit for better detection methodologies of aflatoxins. The need of sensitive detection methods for aflatoxins is faced with challenges of detecting the toxins in complex matrices such as food. We propose to test the sensitivity of detection and quantification limits of these toxins using a quantitative real-time immuno PCR assay. The study will involve developing a sensitive method to detect mycotoxins in raw ingredients (cereals & grains), cooked or processed foods meant for consumption by humans, and in animal feeds.

MATERIALS AND METHODS

Antigens and Antibodies. For the immunoassay, purified aflatoxin standards B1, B2, G1, and G2, monoclonal antibody (mouse igG1 isotype) and polyclonal anti-aflatoxin B1 antibody (developed in rabbit) were purchased from Sigma-Aldrich (St. Louis. MO).

Coating of Paramagnetic Dynabeads.Tosylactivated M-280 dynabeads for immunomagnetic capture of aflatoxins were supplied by Dynal Biotech (Oslo, Norway). The beads were coupled with polyclonal anti-aflatoxin antibodies for antigen capture as described by Alefantis et al., (2004) and by the manufacturer. The coating of beads with antibodies was done using a semi-automatic bead retriever supplied by Dynal Biotech (Oslo, Norway).

Preparation of Reporter DNA using pGL2 Basic Vector. A 563 bp amplimer encoding *luciferase* gene of firefly from pGL2 basic vector (Promega, Madison, WI) was amplified as described by Wu et al., (2001). Briefly, a portion of the *luciferase* gene was amplified using a C6 amino-modified forward primer at 5' end so that the reporter DNA could contain an amino group that can be further used to link the DNA to antibody. PCR amplification of the *luc* gene fragment was done using 0.5μl of the concentrated plasmid (1μg/ml) in a 100 ml of PCR mix containing 2 μM of the pGL2A forward primer (Integrated DNA technologies (IDT), Coralville, IA) and 2 μM the unmodified pGL2A reverse primer (IDT), 0.25 mM dNTPs,

Conjugation of Reporter DNA to Anti-Aflatoxin Antibody. The 5' amino modified end of the reporter DNA (~15ng/μl) was covalently tethered to water soluble Sulfosuccinimidyl-trans-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC) cross linker (Calbiochem, NJ) and the conjugate was purified using a spehadex G-50 column. Sulfide groups of a dilute solution of ($4x10^9$ molecules/μl) polyclonal anti-aflatoxin antibody were reduced using dithiothreitol (DTT), purified and combined with the SMCC linked reporter DNA as described by Hendrickson et al., (1995). The conjugate was used as the detector antibody in further experiments.

Detection and Quantification of Aflatoxin using Realtime Immuno qPCR. Real-time PCR was done by amplifying a 101 bp internal region of the reporter DNA using another set of primers (pGL2B) optimized for qPCR. Standard curve was developed using dilutions of the purified reporter DNA. Paramagnetic dynabeads coated with anti-aflatoxin capture antibody were incubated in known concentrations of the afltoxin-B1 standards at 25^{o}C for 1 h with gentle agitation. The beads were then washed three times with phosphate buffered saline solution using magnetic bead retriever (Dynal) and conjugated with reporter DNA linked detector antibody at 25^{o}C for 2 h. The real-time qPCR assay was done using MJ Research Opticon-2 Real-time PCR detection system for detection and quantification using SYBR Green I absolute quantitative PCR master mix supplied by Abgene (Epsom, UK).

Table 1. Primers used for preparing reporter DNA and real-time PCR

	Primers	**Product**
pGL2A-FW	5'-C6 NH_2-GTTCGTCACATCTCATCTAC-3'	Large amplimer primers for reporter DNA (563bp product).
pGL2A-Rev	5'-TCGGGTGTAATCAGAATAGC-3'	
pGL2B-FW	5'-GAACTGCCTGCGTCAGATTC-3'	Real time qPCR primers (101bp product).
pGL2B-Rev	5'-AACCGTGATGGAATGGAACAAC-3'	

RESULTS AND DISCUSSION

Preparation of Reporter DNA: Reporter DNA prepared using pGL2 basic vector as template was purified and concentrated (Figure 1a) and the SMCC-linked reporter DNA conjugated with anti-aflatoxin antibodies and checked on a agarose gel (Figure 1b).

Detection and Quantification of Reporter DNA and Standard Curve Development using Real-Time PCR: Purified reporter DNA was diluted in 10-folds and subjected to real time PCR using SYBR green chemistry. Amplification plots were developed using the Cycle number and fluorescence and log quantity data. The amplification plots showed that the toxin detection using this approach can go beyond 0.00001 ppb levels indicating the sensitivity of the approach (Figure 2 & 3). Further testing will be done to detect the standard toxins and also the toxins in food and feed matrices.

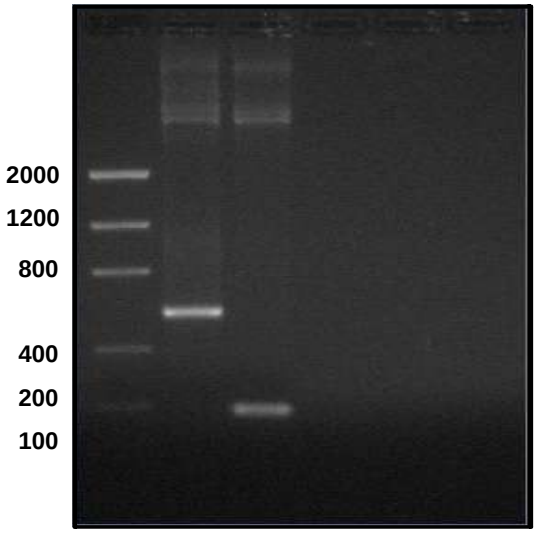

Figure 1a. PCR amplification of *luciferase* gene.

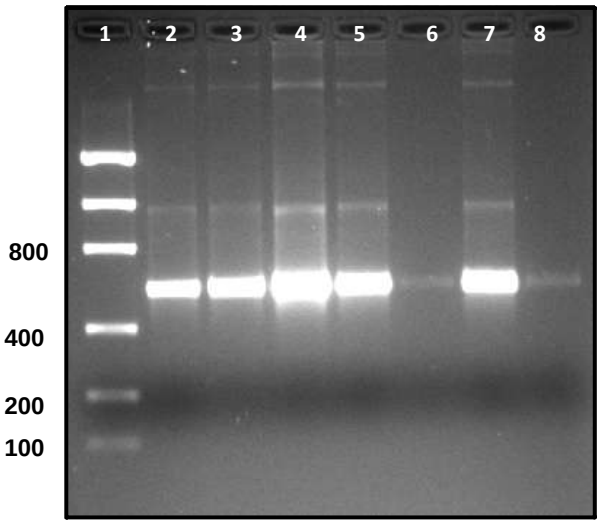

Figure 1b. Antibody coupled reporter DNA.

Lane 1: Low mass DNA ladder
Lane 2: 1:20 diluted reporter DNA (563bp)
Lane 3: SMCC linked reporter DNA before filtration
Lane 4: SMCC linked reporter DNA before filtration
Lane 5: Anti-aflatoxin monoclonal antibody with SMCC linked reporter DNA
Lane 6: 1:100 dilute anti-aflatoxin monoclonal antibody with SMCC linked reporter DNA
Lane 7: Anti-aflatoxin polyclonal antibody with SMCC linked reporter DNA
Lane 8: 1:100 dilute Anti-aflatoxin polyclonal antibody with SMCC linked reporter DNA

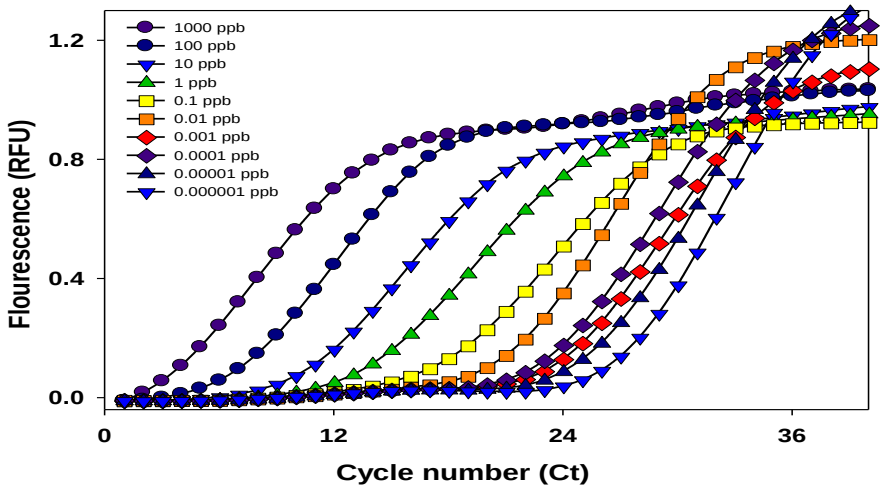

Figure 2. Real-time PCR amplification and detection of reporter DNA standards.

Detection of Aflatoxin Standards using Monoclonal and Polyclonal Capture Antibodies: The sensitivity of our real-time immune qPCR was tested for detecting the standard toxin dilutions of Aflatoxin B1. Four fold dilutions of toxins were subjected to capture by the detection antibodies as described above and an internal region of the reporter DNA was amplified using optimized combinations of pGL2B primers. The detection of aflatoxin B1 concentrations ranging from 0.02

ppb to 400 ppb levels was tested and the results indicated that our approach is sensitive for various concentrations (Figure 4 & 5).

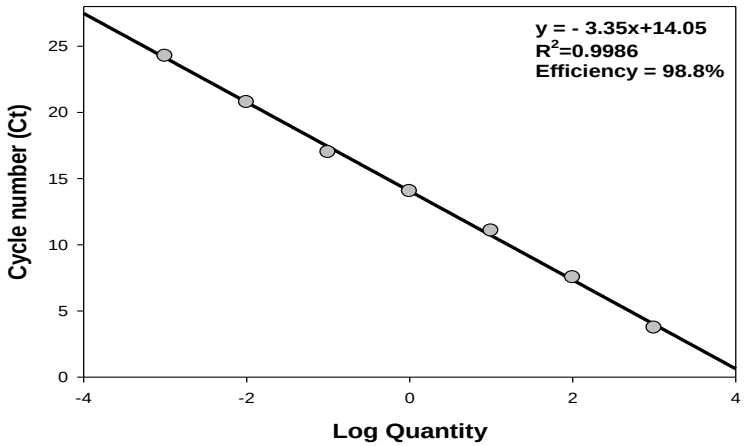

Figure 3. Standard curve developed using 10-fold diluted reporter DNA

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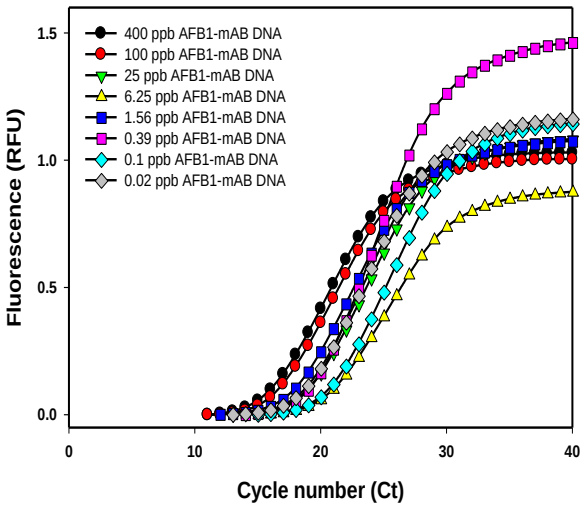

Figure 4. Detection of Aflatoxin B1 standards using Real-time immune qPCR with monoclonal anti-AFB1 antibody for antigen capture.

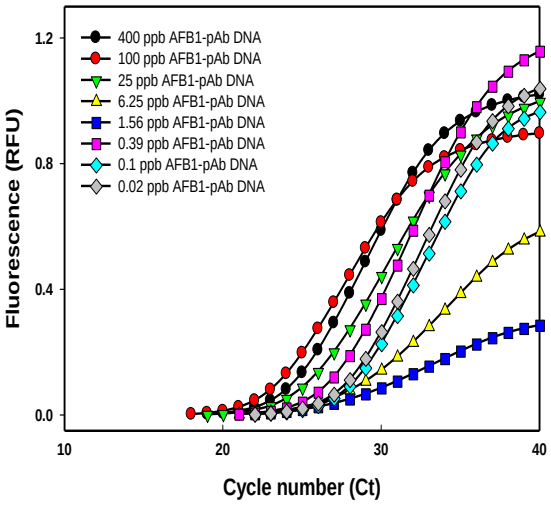

Figure 5. Detection of Aflatoxin B1 standards using Real-time immune qPCR with polyclonal anti-AFB1 antibody for antigen capture.

CONCLUSIONS

The sensitivity and rapidity of paramagnetic bead based immune detection of Aflatoxin B1 was tested in this study and our preliminary results indicated that real-time immune qPCR amplification strategies can successfully be employed for sensitive detection of AflatoxinB1 standards. We propose to be able to detect and quantify the toxins at levels in compliance with US FDA recommendation allowed in food and animal feeds.

LITERATURE CITED

Alefantis, T., P. Grewal, J. Ashton, A. S. Khan, J. J. Valdes, and V. G. Del Vecchio. 2004. A rapid and sensitive magnetic bead- based assay for the detection of staphylococcal enterotoxin B for high through put screening. Mol. Cell Probes. 18(6):379-382.

CAST. 2003. In Richard J. L., Payne G. A. (Eds). Mycotoxins: risks in plant, animal and humans (199 pp). Council of Agricultural Science and Technology Task force report No. 139, Ames, Iowa.

Hendrickson, E. R., T. M. Truby, R. D. Joerger, W. R. Majarian and R. C. Ebersole. 1995. High sensitivity multianalyte immunoassay using covalent DNA- labeled antibodies and polymerase chain reaction. Nucleic Acids Res. 23(3):522-529.

Lynch G. P. 1972. Mycotoxins in feedstuffs and their effect on dairy cattle. J. Dairy Sci. 55 (9):1243-1255.

Wu, H. C., Y. L. Huang, S. C. Lai, Y. Y. Huang, and M. F. Shaio. 2001. Detection of *Clostridium botulinum* neurotoxin type A using immuno PCR. Lett. Appl. Microbiol. 32: 321-325.

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