RAPID AND SENSITIVE DETECTION OF AFLATOXIN IN ANIMAL FEEDS AND FOOD GRAINS USING IMMUNOMAGNETIC BEAD BASED RECOVERY AND REAL-TIME IMMUNO QUANTITATIVE PCR (RT-iqPCR) ASSAY

By

DINESH BABU

Master of Science in Agricultural Microbiology
University of Agricultural Sciences
Bangalore, India
1997

Master of Science in Soil Science
Oklahoma State University
Stillwater, OK. USA
2005

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
December, 2010

RAPID AND SENSITIVE DETECTION OF AFLATOXIN IN ANIMAL FEEDS AND FOOD GRAINS USING IMMUNOMAGNETIC BEAD BASED RECOVERY AND REAL-TIME IMMUNO QUANTITATIVE PCR (RT-iqPCR) ASSAY

Dissertation Approved

Dr. Peter Muriana
Dissertation Adviser

Dr. Christina Dewitt

Dr. Patricia Rayas-Duarte

Dr. William McGlynn

Dr. Mark E. Payton
Dean of the Graduate College

ACKNOWLEDGMENTS

With immense pleasure and deep sense of gratitude, I take this opportunity to thank several outstanding individuals and scholars who guided me throughout the period of my graduate program at Oklahoma State University (OSU). I cherish all the days spent here at Robert M. Kerr Food and Agricultural Product Center (FAPC), OSU.

I feel very fortunate to have been working with Dr. Peter Muriana, my research advisor and mentor. This dissertation would not have been possible without his insight into research projects, guidance, encouragement and support right from the beginning of my doctoral program. I could achieve my research goals through his valuable suggestions and directions. Dr. Muriana always had a new approach of solving every experimental challenge I faced over the years and I thank for his critical suggestions to bring out the best of me. I will always appreciate his confidence in my research capabilities and I am sincerely thankful to him for letting me to work on so many other projects of academic and industry relevance that gave me good exposure to research on food pathogens and food safety aspects.

I would also like to convey my deep sense of appreciation to my research advisory committee members, Dr. Christina DeWitt, Dr. Patricia Rayas-Duarte, and Dr. William McGlynn for their willingness to be on my graduate committee, extending their expert advice in my research and critiquing my dissertation in spite of their busy schedules. Every suggestion the committee members gave me during several interactions with each of them, strengthened my dissertations further.

I sincerely thank Dr. Roy Escoubas and Dr. Ron Kensinger for the opportunity to pursue my graduate program at FAPC and OSU animal sciences department and their efforts in creating excellent research facilities and great working environment. I thank OSU Willard Sparks beef research center, animal science department for providing me with various feed samples for my research. I am also thankful to Dr. Brad Morgan for letting me work with him and his research team in the antimicrobials efficacy project wherein I received several hands on experiences of dealing with issues faced by several meat industries. I enjoyed discussing with Dr. Daniel Stein especially on microarray project and thank him for providing his great inputs. I would also like to extend my sincere thanks to Mr. Darren Scott, Mr. Chuck Willoughby and Dr. Rodney Holcomb and many of my friends who helped me during my product development activities at FAPC. I cherish the friendliness and accommodating nature of the faculty and staff at FAPC. I also appreciate and convey my heartfelt thanks to all my colleagues for their help, support, and friendship.

I am extremely thankful to my wife Kalpana for her constant support and encouragement throughout my study period. She has been a great friend and keeps me going with her everlasting smile in various endeavors in my life. My son Aarush has been a source of relaxation for me and playing with him whenever I got time always filled me with energy. Words are not enough to describe the patience and sacrifice of my parents, In-laws, brothers and sisters back home. Their encouragement will always be cherished.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

Chapter II

Chapter III

ABBREVIATIONS AND SYMBOLS

1. FDA……...……Food and Drug Administration
2. FAO…….Food and Agriculture Organization of the United Nations
3. WHO……..……. World Health Organization
4. CAST……...… Council for Agricultural Science and Technology
5. IARC…….……...International Agency for Research on Cancer
6. U.S. FDA……..United States Food and Drug Administration
7. IAEA……...…….International Agency for Atomic Energy
8. AOAC…….……Association of Agricultural Communities
9. HACCP……Hazard Analysis and Critical Control Point
10. AFB_1……..…….Aflatoxin B1
11. OTA……..……..Ochratoxin A
12. DON…….……..Deoxynivalenol
13. TLC……..…...Thin-layer Chromatography
14. HPLC…….……High-performance liquid chromatography
15. GC……. Gas Chromatography
16. ELISA…..……...…….……Enzyme Linked Immunosorbent Assay
17. PCR…..…….……Polymerase Chain reaction
18. iPCR…….Immuno-PCR
19. RT iqPCR……Real Time immuno quantitative Polymerase Chain Reaction

CHAPTER I

INTRODUCTION

Food safety is an international concern due to the increasing globalization of production and trade of foods contributing to the potential health risks to consumers worldwide. Unsafe food or feeds may involve one or more hazards causing unacceptable health risks. During the last few decades, the concern of animal feed safety has risen due to the potential transfer of certain hazards from unsafe feeds into the food chain and possibly impacting human health. The importance of a hazard in animal feed is selected based on its relevance to public health, extent of occurrence and the impact on trade (FAO/WHO, 2008). Majority of these undesirable substances comprise of chemical (e.g., dioxins, mycotoxins, heavy metals, drug residues, pesticides) and microbiological hazards (e.g., *Salmonella, Brucella*).

Mycotoxins are the secondary metabolites of toxigenic fungi commonly encountered in animal feeds. The infestation of toxigenic fungi can occur before or after harvest of several food crops of grain, oilseeds, fruits and vegetables resulting in serious human and animal health consequences. Mycotoxins are known to contaminate nearly 25% of the world's food crops annually (Mannon and Johnson, 1985; FAO, 2004) and the annual mycotoxin related losses in the United States is estimated to range from $0.5 million to over $1.5 billion (Vardon et al., 2003). The Council for Agricultural Science and Technology (CAST) estimates that crop losses due to mycotoxin contamination of corn, wheat and peanuts amounts to a mean economic annual cost of

$932 million, and an average cost of $466 million from the enforcement of regulations, and quality control measures (CAST, 2003).

Due to the lack of a universal approach to eliminate these fungal toxins and because of their production by several strains of ubiquitous fungi, the mycotoxins are considered as unavoidable natural contaminants of foods and feedstuffs. These toxins are mainly produced by fungal groups of *Aspergillus, Fusarium,* and *Penicilium* whose severity of infestation and toxin production is promoted under favorable conditions of temperature, excessive moisture, relative humidity, drought, insect damage, and variation in crop harvesting practices (Trenk and Hartman, 1970; Bennett and Klich, 2003; Reddy et al., 2005; Abbas et al., 2007; Bircan et al., 2008). There are nearly 400 mycotoxins known, and the important toxins of concern include aflatoxins, fumonisins, ochratoxin A (OTA), deoxynivalenol (DON or vomitoxin), zearalenone, T-2 toxin and T-2 like toxins. Evaluating the carcinogenic potential of several mycotoxins, the World Health Organization (WHO)-International Agency for Research on Cancer has classified aflatoxins as carcinogenic to humans (Group 1), and ochratoxins and fumonisins as possible carcinogens (Group 2B) (IARC, 1993; Vainio and Wilbourn, 1993; Vainio et al., 1994). Among several types of aflatoxins, aflatoxin B_1 (AFB_1) is the most toxic and prevalent member of the group (IARC, 1993; Puschner, 2002; Richard, 2007). Aflatoxins specifically AFB_1 type is known to incur a wide range of adverse acute and chronic toxic effects on human and animal health irrespective of the mode of entry via ingestion or inhalation or dermal contact (Riley et al., 1985; CAST, 2003). The severity of aflatoxin toxicity is mainly dependent on dose, age, duration of feeding and other factors such as stresses affecting an animal. Though there is no safe level of aflatoxins, chronic exposure to low mycotoxin levels can result in reduced productivity and increased susceptibility to infectious diseases (Hussein and Brasel, 2001). In order to avoid ill effects on human and animal health and due to frequent occurrence and associated toxicity of aflatoxins, several countries have set maximum permissible limits in commodities of food and

feeds. These limits for aflatoxin B_1 in human foodstuffs range from as low as 0.05 ppb in European Union to 20 ppb in the United States.

The analytical methods detecting these low permissible limits for aflatoxins need to be specific, sensitive and able to quantify the trace levels. Among several available methods for aflatoxin detection, immunoassay methods are proven to provide such assurance during routine diagnostic applications of aflatoxin detection. Recently, immuno-PCR (iPCR) approaches that combine the advantages of immunoassays with enormous DNA amplification potential of PCR have become popular for sensitive antigen detection. Boasting a 10-to 1,000 fold increase in limit of detection than the ELISA methods (Sano et al., 1992; Niemeyer et al., 2005), immuno-PCR approaches allow quantification of an antigen with greater rapidity and sensitivity. However, the use of this highly sensitive real time immuno-PCR approach has not been exploited as of now to quantitatively determine contamination of mycotoxins such as aflatoxin B_1 in complex matrices of foodstuffs, animal feeds and feed grains. In this study, we demonstrate the use of immuno-PCR assay for sensitive detection and quantification of chronic levels of aflatoxin B_1 in animal feeds and feed grains. The methodology developed here is a simplified noncompetitive sandwich immuno quantitative PCR (RT-iqPCR) approach for detection and quantification of aflatoxin B_1.

CHAPTER II

REVIEW OF THE LITERATURE

Historical perspective of aflatoxins.

The discovery of aflatoxins happened in the late 1950's during an epidemic called turkey "X" disease that caused numerous deaths of turkey poults, ducklings, and chicks in South Eastern England. It was later confirmed that the birds were fed with diets containing Brazilian peanut meal (Blount, 1961). The peanut meal was found to be containing toxic agents produced by a fungus called *Aspergillus flavus*. When fed with the peanut extracts contaminated with *A. flavus*, the toxic agents caused acute liver diseases in ducklings and liver cancer in rats (Lancaster et al., 1961; Sargeant et al., 1961). The toxic substance was thus named as aflatoxin (*A. flavus* toxin) and proved to be the cause of liver cancer in turkey poults causing their death. Massive hepatic necrosis, parenchymal cell degeneration, and bile duct proliferation were also noted in affected poults during the outbreak of turkey "X" disease (Blount, 1961).

Early detection methods showed that aflatoxins emit intense fluorescence in ultraviolet light and consisted of blue (aflatoxin B) and green-fluorescence emitting (aflatoxin G) types (Nesbitt et al., 1962). Subsequent investigations led to the discovery, separation and confirmation of various aflatoxins and their metabolites. The toxins were separated into four closely related compounds of aflatoxin B_1, B_2, G_1 and G_2 (Hartley et al., 1963) and a metabolite of aflatoxin B_1 called M_1 type (Holzapfel et al., 1966). There are nearly 13 different types of aflatoxins produced and among these types, aflatoxin B_1 (AFB_1) is produced as the major metabolite by the aflatoxigenic molds (Heathcote and Hibbert, 1978). Further, chemical structures of aflatoxins

were studied and it was found that the toxins are highly substituted coumarins (Fig.1) containing a fused dihydrofurofuran moiety (Chang et al., 1963; Cheung and Sim, 1964; Asao et al., 1965; Holzapfel et al., 1966; Dutton and Heathcote, 1968). Noticeably, the blue and green-fluorescence properties of aflatoxins discovered in the early 1960's prompted studies of their occurrence in grains and other food commodities.

Toxigenic Aspergillus spp.

The *Aspergillus* is one of the most studied and early named genera of fungi and reported as metabolically and ecologically diverse group of molds. During 1729, Pier Antonio Micheli named the fungi after microscopically observing their spore bearing structures resembling an aspergillum (a brush used for sprinkling holy water in the Roman Catholic Church). Thus, the molds producing the characteristic aspergillum like asexual spore head were classified together in the genus *Aspergillus*. Currently, there are nearly 250 named species of *Aspergillus* (Geiser et al., 2008) classified under the phylum *Ascomycota* (Geiser et al., 2006). Major examples of aspergilli fungi are *A. nidulans, A. fumigatus, A. oryzae, A. parasiticus, A. clavatus, A. flavus, A. niger,* and *A. terreus.* The molds *A. flavus* and *A. parasiticus* are aflatoxigenic and can produce aflatoxins in a majority of foodstuffs and feedstocks. Several other *Aspergillus* fungi producing the toxic metabolites include *Aspergillus nomius, A. pseudotamarii, A. bombycis,* and *A. ochraceoroseus* (Cotty, 1994; Cotty and Bhatnagar, 1994; Bhatnagar et al., 2003).

Occurrence, Identification and Isolation of Aspergillus fungi.

Many of the *Aspergillus* species are ubiquitous, found on diverse substrates such as soil, water, and indoor air and have no strict metabolic requirements. They can grow as saprophytes on decaying vegetation and take part in natural cycling of nutrients. Hot and dry conditions favor the growth of *Aspergillus* fungi such as *A. fumigatus* which can grow at elevated temperatures of

55^{o}C. The fungi can be grown using Czapek dox agar and potato dextrose agar in the laboratory (Hara et al., 1974). Morphologically, the colonies of *Aspergillus* fungi exhibit powdery texture, hyaline and septate hyphae with typical vesicles at their tips. Many of the species can be differentiated based on their characteristic microscopic structures such as sclerotia, cleistothecia, aleuriconidia and the surface color. Identification of the species based on culture characteristics such as surface texture, topography and pigmentation, are not very reliable. Modern methods of detection are usually targeted for species identification and detection of aflatoxin producing species is usually based on ultraviolet detection of diffused aflatoxin into an agar medium (Lin et al., 1976).

Prevention of aflatoxigenic fungi

Current strategies that are in practice for the prevention of aflatoxigenic fungal strains can be mainly categorized as pre-harvest and post-harvest practices. Pre-harvest practices include utilization of non-toxigenic strains of *A. flavus* as biological control agents (Cotty, 2006), use of anti-fungal agents to block molecular interactions of fungi and host plant, and cultivation of plants resistant for fungal infestation (Brown et al., 2001; Campbell et al., 2003). Post-harvest control measures of aflatoxin formation by toxigenic molds mainly include the use of improved storage conditions, achieving optimal drying, and controlling moisture and temperature of the storage facility. Use of chemical preservatives has also been proposed in the absence of proper storage conditions (Al-Hilli and Smith, 1992).

Occurrence of aflatoxins.

Due to several species of fungi capable of producing toxins under diverse growth conditions, wide variety of agricultural commodities worldwide may contain aflatoxins. The most pronounced contamination is often seen in nuts, oilseeds and cereal grains. In general, tropical regions of the world show frequent aflatoxin occurrence than the temperate zones. However, due

to the global trade of agricultural commodities, no region of the world can be considered aflatoxin free (CAST, 2003). Aflatoxins can be encountered in animal feeds and various commodities before or after harvest during storage contaminating commodities through colonization, growth and toxin production. The use of poor quality grains of corn, peanuts and other oilseeds as feed ingredients may cause the prevalence of aflatoxins in animal feeds. Animals fed with unsafe aflatoxin containing feeds, have shown a negative impact on their health and aflatoxin M_1 and M_2 have been found in milk of cows fed with animal rations containing aflatoxin B_1 and B_2 (Holzapfel et al., 1966). Thus, aflatoxins are considered as unavoidable natural contaminants of foods and feedstuffs due to the lack of a single control measure and production by several strains of ubiquitously found toxigenic fungi (CAST, 2003).

Aflatoxin toxicity in animals and humans.

As the contamination of foodstuffs and feedstocks by aflatoxigenic molds and their toxins is universal, the toxic effects of aflatoxins on animal health are encountered worldwide (FAO, 2004). Many animal species of turkeys, ducklings, rainbow trout, guinea pigs, rabbits, rats and dogs show high susceptibility to aflatoxins (Busby and Wogan, 1984; Eaton and Gallagher, 1994). Although the animal species may show variation in their susceptibility to aflatoxins, the severity of aflatoxin toxicity depend on many factors such as dose, age, sex, mode of application, duration of feeding and other factors such as stresses affecting an animal. In general, several research reports agree that aflatoxins are more toxic for young animals (IARC, 1993, Vainio et al., 1994).

With liver being the principal target organ of aflatoxicosis in animals, the first symptoms include lack of appetite and weight loss (Butler, 1964; Barber et al., 1968). The carcinogenic properties of aflatoxin B_1 has been well established in several animal species including rodents, nonhuman primates, and fish (Butler and Barnes, 1968; Canton et al., 1975; Busby and Wogan, 1984; Eaton and Gallagher, 1994). In the case of primates, liver damage is reported as a primary

toxic effect caused by aflatoxins (Tulpule et al., 1964) and much of research information on AFB_1 carcinogenicity is obtained from reports in rats, which are highly susceptible to the toxin (Wogan et al., 1974; Theumer et al., 2009).

The toxicity of AFB_1 occurs in the tissues such as the liver of the affected animal wherein it is converted to a reactive epoxide catalyzed by the mixed function mono-oxygenases belonging to the Cytochrome P450 super family of enzymes (Guengerich et al., 1998). The Cytochrome P450 enzymes may also catalyze the hydroxylation (to AFQ_1 and AFM_1) and demethylation (to AFP_1) of the parent AFB_1 molecule, resulting in products less toxic than AFB_1 (Gross-Steinmeyer et al., 2009; Willard et al., 2009; Zeng et al., 2009). This highly reactive AFB_1 8, 9-epoxide is known to form derivatives with several cellular nucleic acids and proteins. The conjugation of AFB_1 to glutathione (mediated by glutathione S-transferase) and its subsequent excretion is regarded as an important detoxification pathway in animals (Johnson and Guengerich, 1997; Smela et al., 2001). The evidences of epidemiology, clinical trials and experimental findings indicate that acute toxicity of aflatoxin and lethality occur upon exposure to large doses of >6000 mg and chronic carcinogenic effects occur upon exposure to small doses for prolonged periods of time (Groopmann and Thomas, 1999). As per the US FDA's bad bug book, a wide variation in LD_{50} values of aflatoxins is found in the case of animal species tested with single doses. For the majority of animals, the LD_{50} value could range from 0.5 to 10 mg/kg body weight as they respond differently in their susceptibility to the chronic and acute toxicity of aflatoxins. Factors that influence the toxicity of aflatoxins can be environmental, exposure level, and duration of exposure, age, health, and nutritional status of diet (US FDA bad bug book, 1992). Thus, the toxicity of aflatoxins and their seemingly unavoidable occurrence in foods and feeds make the prevention and detoxification of these mycotoxins as the most challenging toxicology problem that needs further study and effective control measures to arrive at accurate detection and prevention strategies.

The toxic effects of aflatoxins (aflatoxicoses) can be distinguished as acute and chronic toxicities based on the duration and dosage of exposure (Leeson et al., 1995). In the case of humans, the exposure to aflatoxins is mainly by consumption of commodities such as corn, peanuts, sorghum, copra and rice, cashew, hazel, peanuts, walnuts, pistachios and almonds (Busby and Wogan, 1984; Abdel-Gawad and Zohri, 1993; Mahoney and Rodriguez, 1996). The levels or final concentrations of aflatoxins in the grain products can vary from less than 1 μg/kg (1 ppb) to 12, 000 μg/kg (12, 000 ppb) or more. Aflatoxin B_1 also causes toxicity through its metabolites like aflatoxin M_1. Aflatoxin M_1 was first discovered to exist in human urine while elucidating the aetiology of liver cancer caused by aflatoxin B_1 (Campbell et al., 1970) and it has been reported that nearly 1.3 to 1.5% of the ingested aflatoxin B_1 is converted to excreted urinary aflatoxin M_1 in humans (Zhu et al., 1987).

Aflatoxin outbreaks.

The foodborne outbreaks associated with aflatoxins are mostly uncommon in developed countries compared to developing countries mainly due to lack of stringent policies, practices, and regulations (Williams et al., 2004). Acute aflatoxin toxicity has been rarely reported in humans. The first incidence of human acute aflatoxicosis occurred during 1967 in the Province of Taiwan due to consumption of rice contaminated with elevated levels of aflatoxin (200 ppb) causing liver necrosis and the death of three children (Ling and Lee, 1967; Ling et al., 1968; Ling, 1976). Another incidence of human aflatoxicosis occurred during 1968 in Canada due to consumption of spaghetti containing high levels of aflatoxin (Van Walbeek et al., 1968; Van Walbeek et al., 1969). The first epidemic of aflatoxicosis affecting humans occurred in India during 1974 affecting nearly 150 villages in the states of Gujarat and Rajasthan. The outbreak was traced to the consumption of maize contaminated with aflatoxin levels of up to 15,000 ppb. More than 100 deaths were reported with several hundred people showing symptoms of aflatoxin poisoning (Krishnamachari et al., 1975a; Krishnamachari et al., 1975b). Aflatoxin containing

maize was also the reason for an acute aflatoxicosis reported in Kenya during 1980 in which 20% mortality was reported (Ngindu et al., 1982). Consumption of contaminated maize was again the cause in one of the largest documented acute aflatoxicosis occurred in rural Kenya during 2004 resulting in 317 cases and 125 deaths (Lewis et al., 2005).

Though there is no safe level of aflatoxins for humans, chronic exposure to lower levels can result in reduced productivity and increased susceptibility to infectious diseases (Hussein and Brasel, 2001). Characterizing dose-response of aflatoxin toxicity, induction of liver tumors in rats was found at various levels of 1, 5, 15, 50 and 100 ppb with incidences of 9, 4.5, 19, 80 and 100% respectively (Wogan et al., 1974). It was an important study that proved chronic toxicity of aflatoxins. Short term exposure to low aflatoxin levels through feeds may cause negligible physiological damage in animals, and long term ingestion of sub-chronic levels can significantly impair the performance of poultry birds (Jones et al., 1982), cause sub-chronic mycotoxicosis in mice (Casado et al., 2001) and rats (Theumer et al., 2009), may lead to impairment of drug-metabolizing enzymes in pig liver (Meissonnier et al., 2007). During 2005 in the United States, more than 75 dogs died along with hundreds showing severe liver problems associated with the consumption of aflatoxin containing pet foods and the U.S Food and Drug Administration (FDA) recalled several batches of nineteen different pet food types contaminated with aflatoxin levels ranging from 61 to 376 ppb (*http://www.fda.gov/AboutFDA/CentersOffices/ORA/ORAElectronicReadingRoom/ucm061767.htm* accessed November 20, 2010).

Regulatory actions for aflatoxin control.

Concern of animal feed safety has risen during last few decades because of the transfer of certain hazards from unsafe feeds into food chain impacting human health. Several regulatory actions levels have thus been set globally for the commerce of food and feed commodities

potentially containing aflatoxins. The first limits of mycotoxins were set during 1960s and by the end of 2003; nearly 100 countries had put forth specific limits for aflatoxins in food and feedstuffs. The United States Food & Drug Administration (FDA) has set the action levels for aflatoxins in various foods and feedstuffs. For example, the action level of 100-300 ppb is set for feeding mature non-lactating animals; 20 ppb for commodities destined for human consumption and interstate commerce; and 0.5 ppb for milk. Any commodities exceeding these levels are not permitted for trade subjected to seizure. Much stricter limitation of 2 ppb for aflatoxin B_1 and 0.05 ppb for aflatoxin M_1 in the foodstuffs have been set by the European Union (Henry et al., 1993). According to an international inquiry by the Food and Agriculture Organization of the United Nations (FAO) conducted during 2002-2003, a median of 5 ppb ranging from 1-20 ppb of maximum tolerated levels was found for AFB_1 in foodstuffs among 61 countries (FAO, 2004). An important observation noted from this inquiry was that though the medians of maximum tolerated aflatoxin levels in foods and feeds remained similar, the range limits for AFB_1 in foodstuffs narrowed from 0-30 ppb during 1995 to 1-20 ppb in 2003. For the animal feeds, the tolerance levels of AFB_1 dropped significantly from 0-1000 ppb in 1995 to 0-50 ppb in 2003. This illustrates the extreme differences in regulatory requirements for aflatoxins among different countries. However, stringent aflatoxin regulatory limits are usually found in developed countries than in developing countries where mycotoxins occur more frequently among the heavily concentrated crops susceptible for aflatoxigenic molds.

Economic impacts of aflatoxins.

Mycotoxins are known to contaminate nearly 25% of the world's food crops annually with a significant economic impact of 1 billion metric tons of food and food product losses annually (Mannon and Johnson, 1985). Majority of the economic losses due to aflatoxin contamination in agricultural commodities may be due to yield losses of crops infested with

toxigenic fungi, reduced crop value, loss of animal productivity due to aflatoxicoses, and monetary losses through health costs. Additional costs of managing the rejected lots, quality testing and crop insurance costs also contribute to the economic impacts. In the United States, the mycotoxin related losses occur mainly as loss of animal health affecting animal producers and cause loss of economic benefits to the feed industry due to rejection of unsafe feeds. The annual mycotoxin related losses in United States is estimated to be ranging from $0.5 million to over $1.5 billion (Vardon et al., 2003). According to the Council for Agricultural Science and Technology (CAST), the estimated crop losses due to mycotoxin contamination of corn, wheat and peanuts amounts to a mean economic annual cost of $932 million, and an average cost of $466 million from the enforcement of regulations, and quality control measures (CAST, 2003). On a global scale, most significant impact of aflatoxins and other mycotoxins may be through the loss of human and animal health along with considerable health care and veterinary costs.

Application of HACCP for mycotoxins.

Through a joint Food and Agriculture Organization of the United Nations and the International Agency for Atomic Energy (FAO/IAEA) training and reference centre for food and pesticide control, a manual on the application of Hazard Analysis and Critical Control Point (HACCP) for mycotoxins has been published in 2001 (FAO/IAEA, 2001). The measures stated in the manual are most widely followed and the control measure include several tasks on hazard analysis, determination of critical control points (CCPs), establishing critical control limits for each CCP, establishing a monitoring system for each CCP, establishing a corrective action, establishing verification procedures and establishing documentation and record keeping.

Hazard analysis task consists of identifying the mycotoxins causing a food safety hazard. By taking the regulatory limits for major mycotoxins into consideration, hazard analysis task identifies a mycotoxin hazard. The risk of a particular mycotoxin hazard is further estimated using the established data on relative susceptibilities of commodities to given mycotoxins and the

climatic conditions required for the mycotoxins to be produced. After identifying a mycotoxin hazard, a commodity flow diagram (CFD) is generated to assess the likelihood of mycotoxin contamination during pre-harvest or post-harvest situations. This will be followed by the introduction of effective control measures such as drying a commodity to lower the water activity (a_w) for prevention of mold growth. Determining a CCP is achieved using decision tree designed by considering each step in a CFD. After this step, critical limits will be set for the acceptable levels at or below the regulatory limits of a particular mycotoxin. Further a monitoring system is established involving a scheduled measurement of a basic parameter such as temperature or time to detect any deviation from the already set critical limits. Corrective actions will be followed in case of any deviation from the critical limits is seen mainly to regain control and to isolate the product out of CCP for further processing or down-grading or discarding the commodity. Verification procedures for the HACCP plan will be established at regular intervals of time to check if the mycotoxin levels in the final product are within acceptable levels followed by standard documentation and record keeping.

Analytical methods for aflatoxins.

Because of the low permissible limits for aflatoxins and the associated high toxicity impacting human and animal health, the analytical methods for determination of aflatoxins need to be sensitive, specific and able to quantify trace levels. Aiming to achieve safety and security of animal feeds and foodstuffs and preventing the associated trade losses, the food and feed industry is in constant pursuit of rapid and reliable methods for detection and quantification of aflatoxins.

The sampling and sample preparation is considered to be a significant source of error contributing to the total variability in any analytical method identifying aflatoxins. This occurs mainly because of the possibility of localized existence of aflatoxins present at extremely high levels in a primary sample (Whitaker and Wiser, 1969). Thus, in order to obtain meaningful analytical results, several key steps of sampling, sample preparation, and analysis are

systematically followed during determination of aflatoxins. Most commonly, any primary sample is well ground and mixed before testing so that the toxin concentrations in the test portions remain close to the original sample. A test sample is usually derived from a large bulk sample and ground during sample preparation step (Rahmani et al., 2009). However, specific plans exist depending on the nature of sample, the testing procedure and the defined limits of acceptance or rejection. For example, FAO/WHO working with Codex Alimentarius Commission has developed an aflatoxin-sampling plan for raw peanuts destined for further processing or trade. The sampling plan outlined consists of use of 20-kg sample which is homogenized and sub-sampled suitably for analytical method.

Chromatography methods.

Numerous chemical methods of aflatoxin analysis in human and animal foodstuffs are described in 'Official Methods of Analysis' published by Association of Agricultural Communities (AOAC). Existing methods involve sample extraction with organic solvent such as methanol, sample clean up and determination. Sample extraction is one or a multi-step process usually performed by use of either liquid-liquid extraction (LLE) involving two immiscible liquid-phase or solid-phase extraction (SPE) involving a solid and liquid phase. During extraction step, the desired analyte and other compounds may migrate into the extractant till equilibrium is achieved enabling the concentration and separation of the desired compounds from any interfering substances present in the sample (Rahmani et al., 2009).

Most frequently used chromatography methods for detection of aflatoxins are; Thin-layer chromatography (TLC), High-performance liquid chromatography (HPLC), and Gas chromatography (GC). Thin-layer chromatography was first used by De Iongh et al., (1964) and it is considered as an AOAC (Association of Agricultural Communities) method since 1990. TLC method is one of the most widely used separation techniques in aflatoxin analysis. In case of TLC methods and most of the liquid chromatography methods, the aflatoxin determination is based on

the intensity of fluorescence emitted by the toxic compounds. Normal-phase TLC involves a stationary phase like silica, alumina, and cellulose immobilized on a glass or plastic plate and a solvent as the mobile phase (Betina, 1985). The TLC method is based on the separation of compounds by their migration distance on specific matrix of silica gel under an organic solvent such as chloroform. The methodology includes application of sample onto TLC plates, plate development, visual observation and quantitation (Trucksess, 1976). In case of a 2-dimensional TLC, the plate is dried after first-development and rotated through 90^{o} to develop again in a different solvent and it is done for better resolution or removal of interfering compounds (Betina, 1985). An advantage of TLC methods is that they can detect more than one mycotoxin for each test sample (Balzer et al., 1978; Trucksess et al., 1984). Several researchers have used the methods of thin-layer chromatography for determination of aflatoxins in various commodities (Allen, 1974; Bodine et al., 1977; Trucksess and Stoloff, 1979; Gulyas, 1985; Abdel-Gawad and Zohri, 1993) and estimations of aflatoxins as low as 1-20 ppb is reported by use of TLC methods (Haddon et al., 1971).

Gas chromatography (GC) is regularly used for mycotoxin identification and quantification in food samples. GC involves detection of volatile products using a flame ionization detector (FID). However most of the mycotoxins are not volatile in nature and need to be derivatised by chemical reactions such as silylation or polyfluroacylation in order to be detected by GC (Scott, 1995). In some cases, the detection of volatile compounds is normally done by linking the GC to a mass spectrometer (MS) or a Fourier transform infrared spectroscopy (FTIR). However, the use of GC is not commonly employed in commercial applications due to the existence of cheaper alternative chromatographic methods for determination of mycotoxins (Liang et al., 2005).

Liquid chromatography (LC) technique is quite similar to TLC method in terms of analyte application, stationary phase, and mobile phase. Liquid chromatography methods for the determination of aflatoxins in foods include normal-phase and reversed-phase liquid

chromatography techniques of HPLC (Rahmani et al., 2009). Stationary phase for HPLC based methods include a C_{18} chromatography column and mobile phases of mixtures of water, methanol or acetonitrile. Early works of Garner (1975) and Seitz (1975) on the use of HPLC methods proved that the aflatoxins can be separated on normal phase columns and detected with either a UV detector or a fluorescence detector. Reversed phase HPLC method is most widely used for separation and determination of and aflatoxins. A programmable fluorescence detector is used for detection and the identification of aflatoxins is done by chemical derivatization. Major advantages of HPLC over TLC are being speed, accuracy, automated analysis, and precision.

Recently, HPLC-MS/MS (Mass spectroscopy) based methods have been used for determination of mycotoxins and their metabolites (Sulyok et al., 2007; Spanjer et al., 2008). The mass spectrometers are not dependent on UV fluorescence or absorbance of an analyte and these methods do not need chemical derivatization of compounds. Further advantages of these methods include ability to generate structural information of the analytes, minimal sample requirement, and low detection limits (Rahmani et al., 2009).

Immunochemical methods.

Due to the inherent disadvantages of laboriousness, time consumption and the need of skills to solve separation and interference problems in analyses using the thin-layer chromatography and liquid chromatography methods, use of immunochemical methods became widely popular during late 1970s. Advances in biotechnology lead to the discovery of antibody-based tests that provided high sensitivity based on the affinities of the monoclonal or polyclonal antibodies for aflatoxins. There are mainly three types of immunochemical methods of radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and immunoaffinity column assay (ICA). Usage of immunochemical methods for determining AFB_1 in peanuts was reported by Langone and Van Vunakis, (1976) using solid phase radio-immunoassay (RIA) and several other workers also reported use of monoclonal antibody affinity columns and enzyme

linked immunosorbent assay (ELISA) techniques for determining aflatoxins in peanuts (Groopman et al., 1982; Lawellin et al., 1977; Chu et al., 1988; Mortimer et al., 1988; Wilkinson et al., 1988; Trucksess et al., 1989). The advantages of ELISA and monoclonal affinity column methods included ease of sample preparation, usage and low cost. However, these methods involve disadvantages of antibody cross reactivity with different types of aflatoxins, temperature sensitivity and their ability to provide only the qualitative or semi-quantitative results (Wilson, 1989).

Several commercially available ELISA kits exist for detection of mycotoxins and they are normally based on a competitive immunoassay format that uses either a toxin specific primary antibody or an enzyme conjugated secondary antibody. In general, the performance of immunoassays depend mainly on the use of molecular label markers such as fluorescent compounds, enzymes, radio isotopes or other signal markers, the format of the assays with competitive or indirect antigen detection, and the type and sensitivity of devices used for signal measurement. Majority of ELISA immunoassays using horseradish peroxidase (HRP) and alkaline phosphatase (AP) enzyme labels are employed in the enzyme-linked immunoassays (ELISA) used in monitoring agricultural commodities for aflatoxins (Pestka et al., 1980; Pestka et al., 1983; Park et al., 1989).

Immuno-PCR methods.

Immuno-PCR (iPCR) methods offer most sensitive antigen detection and they are not yet explored for aflatoxin detection and quantification. These methods combine the advantages of immunoassays with the enormous DNA amplification potential of PCR. In case of immunoassays such as ELISA, detection of few million analyte molecules per milliliter is possible. However, many cellular proteins, diagnostic markers, drug targets and other biomolecules may be present below the threshold of ELISA methods. Using DNA as the amplifiable marker, iPCR typically leads to a 10 to 1,000 fold increase in the limit of detection over ELISA methods (Sano et al.,

1992) allowing the quantification of an antigen with greater rapidity and sensitivity. Immuno-PCR methods involve use of highly specific primary antibodies for antigen capture and a secondary antibody conjugated with DNA as signal generating marker (Sano et al., 1992; Niemeyer and Blohm, 1996; Adler, 2005; Niemeyer et al., 2007). Thus, the enzyme label in immunoassay is replaced by DNA in an immuno-PCR method which by PCR amplification can detect very low copy numbers of DNA. Use of real time-PCR for DNA amplification has further increased the sensitivity of iPCR methods with rapidity and sensitivity beyond conventional PCR methods (Fischer et al., 2004; Zhang et al., 2008; Adler and Eisenbraun, 2007; Chen and Zhuang, 2009). Since its introduction by Sano et al., in 1992, several immuno-PCR approaches have been developed for detection of various high molecular weight antigens. Several researchers have demonstrated the use of highly sensitive iPCR in a sandwich immunoassay format wherein a capture antibody is coated on the surface of a microtiter well or a bead as solid supports (Joerger et al., 1995). Several other formats of iPCR have been explored by researchers using two-antibody sandwich formats for the detection of several antigens with significant improvements over conventional sandwich ELISA methods (Hendrickson et al., 1995).

Aflatoxin detection has largely relied on competitive ELISA formats that are semi-quantitative and depend on the use of conjugated toxin molecules. However, the limitation of conventional immunoassay formats is largely due to the requirement that at least two epitopes of an antigen to be recognized and occupied (Lim et al., 2007). It is more challenging to develop a sandwich immunoassay assay for low molecular weight hapten molecules such as aflatoxins since the antibodies are much larger in size (150 K Da) than the aflatoxin molecules (~0.312 K Da) themselves preventing combinatorial association of antibodies due to steric hindrance and limiting access of the antigen to secondary antibodies. It is also reported that the sandwich immunoassay for molecules of 1000 Daltons MW is less amenable for such applications (Lim et al., 2007; Quinton et al., 2010). However, sandwich immunoassays for homovanillic acid (MW 182.17; (Quinton et al., 2010) and open sandwich ELISA for angiotensin II (MW 1046.18; (Lim

et al., 2007) have been conducted to prove that low molecular weight molecules can be used successfully in sandwich immunoassays. However, it is unclear about how small a hapten may be to allow simultaneous recognition by two different antibodies and may be a principle reason why the use of sensitive real-time immuno-PCR has not been exploited to quantitatively determine mycotoxins such as aflatoxin B_1.

Considering the significance of estimating trace levels of aflatoxins and their serious implications on animal and human health, it is important to obtain develop and test a quantitative real time immuno-PCR method that can be used as a model to detect mycotoxins to meet with stricter regulatory limits in food and feedstuffs. To evaluate the challenges and various immuno-PCR approaches, the following objectives were laid out in this study.

Objective 1: Development of a sensitive and reliable immuno-PCR technique that can detect and quantify aflatoxins in organic solvent extractions.

Objective 2: Evaluation of suitable immuno-PCR approach for detecting and quantifying aflatoxin B_1 in complex matrices of foods, feedstuffs and feed ingredients.

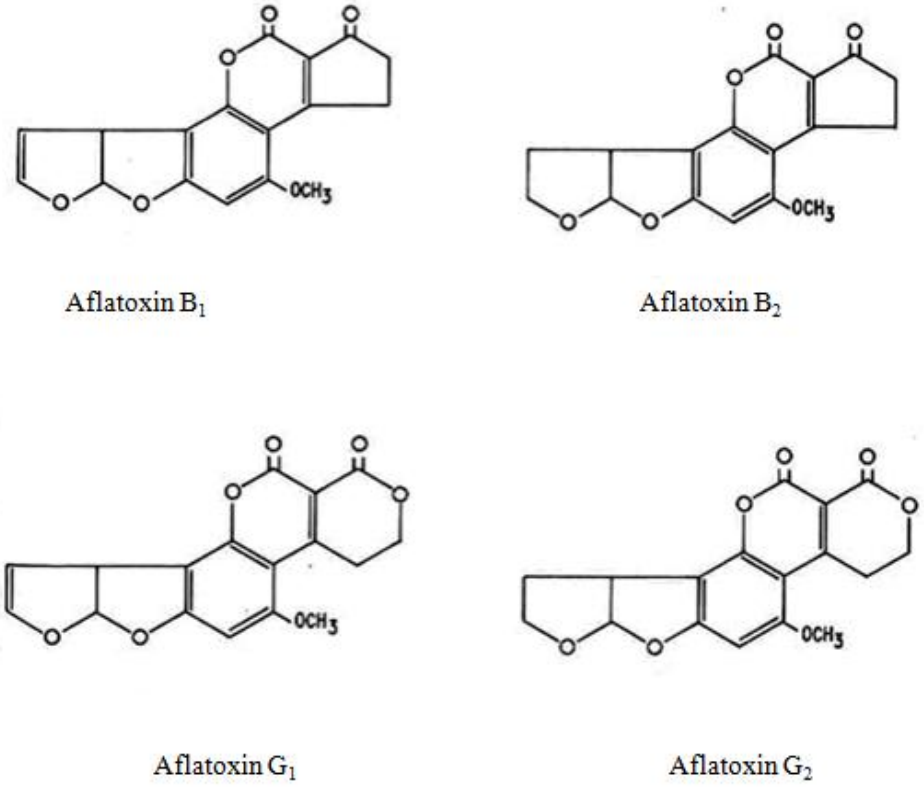


Figure 1. Chemical structures of the four naturally occurring aflatoxins B_1, B_2, G_1, and G_2. These aflatoxins are mainly produced by *Aspergillus* spp. and the letters B and G denote for the characteristic emission of fluorescence under UV light as blue and green respectively.

References

Abbas, H.K., Shier, W.T. and Cartwright, R.D. (2007) Effect of temperature, rainfall and planting date on aflatoxin and fumonisin contamination in commercial Bt and non-Bt corn hybrids in Arkansas. Phytoprotection 88, 41.

Abdel-Gawad, K.M. and Zohri, A.A. (1993) Fungal flora and mycotoxins of six kinds of nut seeds for human consumption in Saudi Arabia. Mycopathologia 124, 55.

Adler, M. (2005) Immuno-PCR as a clinical laboratory tool. Advances in Clinical Chemistry, Vol. 39, 239.

Adler, M. and Eisenbraun, J. (2007) Immuno-PCR-highly sensitive protein detection: Results of the detection of native mistletoe lectin in human serum samples. Phytomedicine 14.

Al-Hilli, A.L. and Smith, J.E. (1992) Influence of propionic acid on growth and aflatoxin production by *Aspergillus flavus* in liquid submerged and solid substrate conditions. J Environ Pathol Toxicol Oncol 11, 57.

Allen, L. (1974) Two-dimensional thin layer chromatography of aflatoxins in figs. J Assoc Of Anal Chem 57, 1398.

Asao, T., Buechi, G., Abdel-Kader, M.M., Chang, S.B., Wick, E.L. and Wogan, G.N. (1965) The structures of aflatoxins b and g. J Am Chem Soc 87, 882.

Balzer, I., Bogdanic, C. and Pepeljnjak, S. (1978) Rapid thin layer chromatographic method for determining aflatoxin B_1, ochratoxin a, and zearalenone in corn. J Assoc Of Anal Chem 61, 584.

Barber, R.S., Braude, R., Mitchell, K.G., Harding, J.D., Lewis, G. and Loosmore, R.M. (1968) The effects of feeding toxic groundnut meal to growing pigs and its interaction with high-copper diets. Br J Nutr 22, 535.

Bennett, J.W. and Klich, M. (2003) Mycotoxins. Clin Microbiol Rev 16, 497.

Betina, V. (1985) Thin-layer chromatography of mycotoxins. J Chromatogr 334, 211.

Bhatnagar, D., Ehrlich, K.C. and Cleveland, T.E. (2003) Molecular genetic analysis and regulation of aflatoxin biosynthesis. Appl. Microbiol. Biotechnol. 61, 83.

Bircan, C., Barringer, S.A., Ulken, U. and Pehlivan, R. (2008) Increased aflatoxin contamination of dried figs in a drought year. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 25, 1400.

Blount, W.P. (1961) Turkey "X" Disease. Journal of the British Turkey Federation 9, 55.

Bodine, A.B., Davis, R.W., Janzen, J.J. and Crawford, F.M. (1977) Comparison of two methods for analysis of aflatoxin M_1 in milk. J Dairy Sci 60, 450.

Brown, R.L., Chen, Z.Y., Menkir, A., Cleveland, T.E., Cardwell, K., Kling, J. and White, D.G. (2001) Resistance to aflatoxin accumulation in kernels of maize inbreds selected for ear rot resistance in west and central Africa. J Food Prot 64, 396.

Busby, W.F., Jr. and Wogan, G.N. (1984) Aflatoxins. ACS Monogr. 182, 945.

Butler, W.H. (1964) Acute toxicity of aflatoxin b-1 in rats. Br J Cancer 18, 756.

Butler, W.H. and Barnes, J.M. (1968) Carcinogenic action of groundnut meal containing aflatoxin in rats. Food Cosmet Toxicol 6, 135.

Campbell, B.C., Molyneux, R.J. and Schatzki, T.F. (2003) Current research on reducing pre- and post-harvest aflatoxin contamination of U.S. Almond, Pistachio, and Walnut. J. Toxicol., Toxin Rev. 22, 225.

Campbell, T.C., Caedo, J.P., Jr., Bulatao-Jayme, J., Salamat, L. and Engel, R.W. (1970) Aflatoxin M_1 in human urine. Nature 227, 403.

Canton, J.H., Kroes, R., van Logten, M.J., van Schothorst, M., Stavenuiter, J.F. and Verhulsdonk, C.A. (1975) The carcinogenicity of aflatoxin M_1 in rainbow trout. Food Cosmet Toxicol 13, 441.

Casado, J.M., Theumer, M., Masih, D.T., Chulze, S. and Rubinstein, H.R. (2001) Experimental subchronic mycotoxicoses in mice: Individual and combined effects of dietary exposure to fumonisins and aflatoxin B_1. Food Chem. Toxicol. 39, 579.

CAST. (2003) Mycotoxins: Risks in plant, animal, and human systems. Task Force Report - Council for Agricultural Science and Technology, xvi + 199 pp.

Chang, S.B., Abdel-Kader, M.M., Wick, E.L. and Wogan, G.N. (1963) Aflatoxin B_2: Chemical identity and biological activity. Science 142, 1191.

Chen, H. and Zhuang, H. (2009) Real-time immuno-PCR assay for detecting pcbs in soil samples. Anal. Bioanal. Chem. 394, 1205.

Cheung, K.K. and Sim, G.A. (1964) Aflatoxin G_1: Direct determination of the structure by the method of isomorphous replacement. Nature 201, 1185.

Chu, F.S., Lee, R.C., Trucksess, M.W. and Park, D.L. (1988) Evaluation of enzyme-linked immunosorbent assay of cleanup for thin-layer chromatography of aflatoxin B_1 in corn, peanuts, and peanut butter. J Assoc Of Anal Chem 71, 953.

Cotty, P.J. (1994) Comparison of four media for the isolation of *Aspergillus* flavus group fungi. Mycopathologia 125, 157.

Cotty, P.J. (2006) Biocompetitive exclusion of toxigenic fungi. Mycotoxin Factbook, [Pap. Conf. World Mycotoxin Forum], 3rd, 179.

Cotty, P.J. and Bhatnagar, D. (1994) Variability among aflatoxigenic *Aspergillus flavus* strains in ability to prevent aflatoxin contamination and production of aflatoxin biosynthetic pathway enzymes. Appl Environ Microbiol 60, 2248.

De Iongh, H., Vles, R.O. and Van, P. (1964) Milk of mammals fed an aflatoxin-containing diet. Nature 202, 466.

Dutton, M.F. and Heathcote, J.G. (1968) The structure, biochemical properties and origin of the aflatoxins B_2a and G_2a. Chem Ind 13, 418.

Eaton, D.L. and Gallagher, E.P. (1994) Mechanisms of aflatoxin carcinogenesis. Annu Rev Pharmacol Toxicol 34, 135.

FAO (2001) Manual on the application of the haccp system in mycotoxin prevention and control. FAO Food Nutr. Pap., vii + 113 pp.

FAO (2004) Worldwide regulations for mycotoxins in food and feed in 2003. FAO Food Nutr. Pap., 1.

FAO/WHO (2008) Joint FAO/WHO expert committee report. In: Animal feed impact on food safety, FAO Headquarters, Rome, 8-12 October 2007. 54.

Fischer, A., Zhang, W.L., Kuczius, T., von Eiff, C., Peters, G. and Becker, K. (2004) Development of a highly sensitive real-time immuno-PCR method for detection of staphylococcal enterotoxin a (sea). International Journal of Medical Microbiology 294, 142.

Garner, R.C. (1975) Aflatoxin separation by high-pressure liquid chromatography. J Chromatogr 103, 186.

Geiser, D.M., Gueidan, C., Miadlikowska, J., Lutzoni, F., Kauff, F., Hofstetter, V., Fraker, E., Schoch, C.L., Tibell, L., Untereiner, W.A. and Aptroot, A. (2006) Eurotiomycetes: Eurotiomycetidae and chaetothyriomycetidae. Mycologia 98, 1053.

Geiser, D.M., Samson, R.A., Varga, J., Rokas, A. and Witiak, S.M. (2008) A review of molecular phylogenetics in *Aspergillus*, and prospects for a robust genus-wide phylogeny. In: *Aspergillus* in the genomic era. Wageningen Academic Publisher, Netherlands.

Groopman, J.D., Haugen, A., Goodrich, G.R., Wogan, G.N. and Harris, C.C. (1982) Quantitation of aflatoxin B_1-modified DNA using monoclonal antibodies. Cancer Res 42, 3120.

Groopmann, J.D. and Thomas, W.K. (1999) *CRC critical reviews in toxicology:* Chapter 19. 113-124.

Gross-Steinmeyer, K., Stapleton, P.L., Tracy, J.H., Bammler, T.K., Strom, S.C., Buhler, D.R. and Eaton, D.L. (2009) Modulation of aflatoxin B_1-mediated genotoxicity in primary cultures of human hepatocytes by diindolylmethane, curcumin, and xanthohumols. Toxicol Sci 112, 303.

Guengerich, F.P., Johnson, W.W., Shimada, T., Ueng, Y.F., Yamazaki, H. and Langouet, S. (1998) Activation and detoxication of aflatoxin B_1. Mutat Res 402, 121.

Gulyas, H. (1985) [determination of aflatoxins B_1, B_2, G_1, G_2 and M_1 by high pressure thin layer chromatography]. J Chromatogr 319, 105.

Haddon, W.F., Wiley, M. and Waiss, A.C., Jr. (1971) Aflatoxin detection by thin-layer chromatography--mass spectrometry. Anal Chem 43, 268.

Hara, S., Fennell, D.I. and Hesseltine, C.W. (1974) Aflatoxin-producing strains of *Aspergillus flavus* detected by fluorescence of agar medium under ultraviolet light. Appl Microbiol 27, 1118.

Hartley, R.D., Nesbitt, B.F. and O'Kelly, J. (1963) Toxic metabolites of *Aspergillus flavus*. Nature 198, 1056.

Heathcote, J.G. and Hibbert, J.R. (1978) Developments in food science, no. 1: Aflatoxins: Chemical and biological aspects.

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

Henry, S.H., Scheuplein, R.J., Bowers, J. and Tollefson, L. (1993) U.S. Food and drug administration approach to risk assessment of aflatoxin in human foods. Qual. Assur. (San Diego) 2, 71.

Holzapfel, C.W., Steyn, P.S. and Purchase, I.F. (1966) Isolation and structure of aflatoxins M_1 and M_2. Tetrahedron Lett 25, 2799.

Hussein, H.S. and Brasel, J.M. (2001) Toxicity, metabolism, and impact of mycotoxins on humans and animals. Toxicology 167, 101.

IARC. (1993) Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. IARC monographs on the evaluation of carcinogenic risks to humans. In, Vol. 56. International Agency for Research on Cancer (IARC) Working Group, World Health Organization, Lyon, France, p. 467.

Joerger, R.D., Truby, T.M., Hendrickson, E.R., Young, R.M. and Ebersole, R.C. (1995) Analyte detection with DNA-labeled antibodies and polymerase chain-reaction. Clinical Chemistry 41, 1371.

Johnson, W.W. and Guengerich, F.P. (1997) Reaction of aflatoxin B_1 exo-8, 9-epoxide with DNA: Kinetic analysis of covalent binding and DNA-induced hydrolysis. Proc Natl Acad Sci U S A 94, 6121.

Jones, F.T., Hagler, W.H. and Hamilton, P.B. (1982) Association of low-levels of aflatoxin in feed with productivity losses in commercial broiler operations. Poult Sci 61, 861.

Krishnamachari, K.A., Bhat, R.V., Nagarajan, V. and Tilak, T.B. (1975a) Hepatitis due to aflatoxicosis. An outbreak in western india. Lancet 1, 1061.

Krishnamachari, K.A., Bhat, R.V., Nagarajan, V. and Tilak, T.B. (1975b) Investigations into an outbreak of hepatitis in parts of western India. Indian J Med Res 63, 1036.

Lancaster, M.C., Jenkins, F.P. and Philp, J.M. (1961) Toxicity associated with certain samples of groundnuts. Nature 192, 1095.

Langone, J.J. and Van Vunakis, H. (1976) Aflatoxin B_1 specific antibodies and their use in radioimmunoassay. J Natl Cancer Inst 56, 591.

Lawellin, D.W., Grant, D.W. and Joyce, B.K. (1977) Enzyme-linked immunosorbent analysis for aflatoxin B_1. Appl Environ Microbiol 34, 94.

Leeson, S., Diaz , G.J. and Sumers, J.D. (1995) Poultry metabolic disorders and mycotoxins. University books, Guelph, ON, Canada.

Lewis, L., Onsongo, M., Njapau, H., Schurz-Rogers, H., Luber, G., Kieszak, S., Nyamongo, J., Backer, L., Dahiye, A.M., Misore, A., DeCock, K., Rubin, C., Nyikal, J., Njuguna, C., Langar, A., Kilei, I.K., Tetteh, C., Likimani, S., Oduor, J., Nzioki, D., Kamau, B.W., Onsongo, J., Slutsker, L., Mutura, C., Mensah, P., Kessel, F., Park, D.L., Trujillo, S., Funk, A., Gieseker, K.E., Azziz-Baumgartner, E. and Gupta, N. (2005) Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in eastern and central kenya. Environ. Health Perspect. 113, 1763.

Liang, Y., Zhang, C. and Liu, L. (2005) Chromatographic analysis of mycotoxins. Zhongguo Weisheng Jianyan Zazhi 15, 1273.

Lim, S.L., Ichinose, H., Shinoda, T. and Ueda, H. (2007) Noncompetitive detection of low molecular weight peptides by open sandwich immunoassay. Anal. Chem. 79, 6193.

Lin, M.T., Mahajan, J.R., Dianese, J.C. and Takatsu, A. (1976) High production of kojic acid crystals by *Aspergillus parasiticus* unbf a12 in liquid medium. Appl Environ Microbiol 32, 298.

Ling, K.-H. (1976) Study on mycotoxins. Contamination of food in taiwan. 2. Tremor-inducing compounds from *Aspergillus terresus*. Proc. Natl. Sci. Counc., Part 2 (Taiwan) 9, 121.

Ling, K.H. and Lee, H.T. (1967) Isolation of a fluorescent compound from *Aspergillus flavus*. Journal of the Formosan Medical Association 66, 182.

Ling, K.H., Tung, C.M., Sheh, I.F., Wang, J.J. and Tung, T.C. (1968) Aflatoxin B_1 in unrefined peanut oil and peanut products in taiwan. Taiwan Yi Xue Hui Za Zhi 67, 309.

Mahoney, N.E. and Rodriguez, S.B. (1996) Aflatoxin variability in Pistachios. Appl Environ Microbiol 62, 1197.

Mannon, J. and Johnson, E. (1985) Fungi down on the farm. New Scientist, 12.

Meissonnier, G.M., Laffitte, J., Loiseau, N., Benoit, E., Raymond, I., Pinton, P., Cossalter, A.M., Bertin, G., Oswald, I.P. and Galtier, P. (2007) Selective impairment of drug-metabolizing enzymes in pig liver during subchronic dietary exposure to aflatoxin B_1. Food Chem Toxicol 45, 2145.

Mortimer, D.N., Shepherd, M.J., Gilbert, J. and Clark, C. (1988) Enzyme-linked immunosorbent (ELISA) determination of aflatoxin B_1 in peanut butter: Collaborative trial. Food Addit Contam 5, 601.

Nesbitt, B.F., O'Kelly, J., Sargeant, K. and Sheridan, A. (1962) Toxic metabolites of *Aspergillus flavus*. Nature 195, 1062.

Ngindu, A., Johnson, B.K., Kenya, P.R., Ngira, J.A., Ocheng, D.M., Nandwa, H., Omondi, T.N., Jansen, A.J., Ngare, W., Kaviti, J.N., Gatei, D. and Siongok, T.A. (1982) Outbreak of acute hepatitis caused by aflatoxin poisoning in kenya. Lancet 1, 1346.

Niemeyer, C. and Blohm, D. (1996) Protein detection with PCR (immuno-PCR). Nachrichten Aus Chemie Technik Und Laboratorium 44, 481.

Niemeyer, C.M., Adler, M. and Wacker, R. (2007) Detecting antigens by quantitative immuno-PCR. Nature Protocols 2, 1918.

Park, D.L., Miller, B.M., Hart, L.P., Yang, G., McVey, J., Page, S.W., Pestka, J. and Brown, L.H. (1989) Enzyme-linked immunosorbent assay for screening aflatoxin B_1 in cottonseed products and mixed feed: Collaborative study. J Assoc Off Anal Chem 72, 326.

Pestka, J.J., Beery, J.T. and Chu, F.S. (1983) Indirect immunoperoxidase localization of aflatoxin B_1 in rat liver. Food Chem Toxicol 21, 41.

Pestka, J.J., Gaur, P.K. and Chu, F.S. (1980) Quantitation of aflatoxin B_1 and aflatoxin B_1 antibody by an enzyme-linked immunosorbent microassay. Appl Environ Microbiol 40, 1027.

Puschner, B. (2002) Mycotoxins. Vet Clin North Am Small Anim Pract 32, 409.

Quinton, J., Charruault, L., Nevers, M.C., Volland, H., Dognon, J.P., Creminon, C. and Taran, F. (2010) Toward the limits of sandwich immunoassay of very low molecular weight molecules. Anal. Chem. 82, 2536.

Rahmani, A., Jinap, S. and Soleimany, F. (2009) Qualitative and quantitative analysis of mycotoxins. Comprehensive Reviews in Food Science and Food Safety 8, 202.

Reddy, B.N., Brijitha, N. and Raghavender, C.R. (2005) Aflatoxin contamination in insect damaged seeds of horsegram under storage. Mycotoxin Res. 21, 187.

Richard, J.L. (2007) Some major mycotoxins and their mycotoxicoses-an overview. Int. J. Food Microbiol. 119, 3.

Riley, R.T., Kemppainen, B.W. and Norred, W.P. (1985) Penetration of aflatoxins through isolated human epidermis. J Toxicol Environ Health 15, 769.

Sano, T., Smith, C.L. and Cantor, C.R. (1992) Immuno-PCR - very sensitive antigen-detection by means of specific antibody-DNA conjugates. Science 258, 120.

Sargeant, K., Sheridan, A., O'Kelly, J. and Carnaghan, R.B.A. (1961) Toxicity associated with certain samples of groundnuts. Nature 192, 1096.

Scott, P.M. (1995) Mycotoxin methodology. Food Addit Contam 12, 395.

Seitz, L.M. (1975) Comparison of methods for aflatoxin analysis by high-pressure liquid chromatography. J Chromatogr 104, 81.

Smela, M.E., Currier, S.S., Bailey, E.A. and Essigmann, J.M. (2001) The chemistry and biology of aflatoxin B_1: From mutational spectrometry to carcinogenesis. Carcinogenesis 22, 535.

Spanjer, M.C., Rensen, P.M. and Scholten, J.M. (2008) Lc-ms/ms multi-method for mycotoxins after single extraction, with validation data for peanut, pistachio, wheat, maize, cornflakes, raisins and figs. Food Addit. Contam., Part A 25, 472.

Sulyok, M., Krska, R. and Schuhmacher, R. (2007) A liquid chromatography/tandem mass spectrometric multi-mycotoxin method for the quantification of 87 analytes and its application to semi-quantitative screening of moldy food samples. Anal. Bioanal. Chem. 389, 1505.

Theumer, M.G., Canepa, M.C., Lopez, A.G., Mary, V.S., Dambolena, J.S. and Rubinstein, H.R. (2009) Subchronic mycotoxicoses in Wistar Rats: Assessment of the in vivo and in vitro genotoxicity induced by fumonisins and aflatoxin B_1, and oxidative stress biomarkers status. Toxicology 268, 104.

Trenk, H.L. and Hartman, P.A. (1970) Effects of moisture content and temperature on aflatoxin production in corn. Appl Microbiol 19, 781.

Trucksess, M.W. (1976) Derivatization procedure for identification of aflatoxin M_1 on a thin layer chromatogram. J Assoc Off Anal Chem 59, 722.

Trucksess, M.W. and Stoloff, L. (1979) Extraction, cleanup, and quantitative determination of aflatoxins B_1 and M_1 in beef liver. J Assoc Off Anal Chem 62, 1080.

Trucksess, M.W., Brumley, W.C. and Nesheim, S. (1984) Rapid quantitation and confirmation of aflatoxins in corn and peanut butter, using a disposable silica gel column, thin layer chromatography, and gas chromatography/mass spectrometry. J Assoc Off Anal Chem 67, 973.

Trucksess, M.W., Stack, M.E., Nesheim, S., Park, D.L. and Pohland, A.E. (1989) Enzyme-linked immunosorbent assay of aflatoxins B_1, B_2, and G_1 in corn, cottonseed, peanuts, peanut butter, and poultry feed: Collaborative study. J Assoc Off Anal Chem 72, 957.

Tulpule, P.G., Madhavan, T.V. and Gopalan, C. (1964) Effect of feeding aflatoxin to young monkeys. Lancet 1, 962.

USFDA (1992) US Food & Drug Administration Center for Food Safety & Applied Nutrition. In: Foodborne Pathogenic Microorganisms and Natural Toxins (Bad Bug Book).

Vainio, H. and Wilbourn, J. (1993) Cancer etiology: Agents causally associated with human cancer. Pharmacol Toxicol 72 Suppl 1, 4.

Vainio, H., Heseltine, E. and Wilbourn, J. (1994) Priorities for future IARC monographs on the evaluation of carcinogenic risks to humans. Environ Health Perspect 102, 590.

Van Walbeek, W., Clademenos, T. and Thatcher, F.S. (1969) Influence of refrigeration on aflatoxin production by strains of *Aspergillus flavus*. Can J Microbiol 15, 629.

Van Walbeek, W., Scott, P.M. and Thatcher, F.S. (1968) Mycotoxins from food-borne fungi. Can J Microbiol 14, 131.

Vardon, P., McLaughlin, C. and Nardinelli, C. (2003) Potential economic costs of mycotoxins in the united states. In: Council for Agricultural Science and Technology (CAST). *Mycotoxins: Risks in Plant, Animal, and Human Systems.* Task Force Report No. 139, Ames, Iowa.

Whitaker, T.B. and Wiser, E.H. (1969) Theoretical investigations into the accuracy of sampling shelled peanuts for aflatoxin. J Am Oil Chem Soc 46, 377.

Wilkinson, A.P., Denning, D.W. and Morgan, M.R. (1988) An ELISA method for the rapid and simple determination of aflatoxin in human serum. Food Addit Contam 5, 609.

Willard, R.R., Shappell, N.W., Meekin, J.H., Talbot, N.C. and Caperna, T.J. (2009) Cytochrome p450 expression profile of the PICM-19H pig liver cell line: Potential application to rapid liver toxicity assays. In Vitro Cell Dev Biol Anim.

Williams, J.H., Phillips, T.D., Jolly, P.E., Stiles, J.K., Jolly, C.M. and Aggarwal, D. (2004) Human aflatoxicosis in developing countries: A review of toxicology, exposure, potential health consequences, and interventions. Am. J. Clin. Nutr. 80, 1106.

Wilson, D.M. (1989) Analytical methods for aflatoxins in corn and peanuts. Arch Environ Contam Toxicol 18, 308.

Wogan, G.N., Paglialunga, S. and Newberne, P.M. (1974) Carcinogenic effects of low dietary levels of aflatoxin B_1 in rats. Food Cosmet Toxicol 12, 681.

Zeng, R.S., Wen, Z., Niu, G., Schuler, M.A. and Berenbaum, M.R. (2009) Enhanced toxicity and induction of cytochrome p450s suggest a cost of "Eavesdropping" In a multitrophic interaction. J Chem Ecol 35, 526.

Zhang, W.L., Bielaszewska, M., Pulz, M., Becker, K., Friedrich, A.W., Karch, H. and Kuczius, T. (2008) New immuno-PCR assay for detection of low concentrations of Shiga toxin 2 and its variants. J. Clin. Microbiol. 46, 1292.

Zhu, J.Q., Zhang, L.S., Hu, X., Xiao, Y., Chen, J.S., Xu, Y.C., Fremy, J. and Chu, F.S. (1987) Correlation of dietary aflatoxin B_1 levels with excretion of aflatoxin M_1 in human urine. Cancer Res 47, 1848.

CHAPTER III

REAL TIME IMMUNO-QUANTITATIVE PCR (RT iq-PCR) APPROACHES FOR RAPID AND SENSITIVE QUANTIFICATION OF AFLATOXIN B1 USING IMMUNOMAGNETIC BEAD BASED RECOVERY

Introduction

Aflatoxins are one of the most commonly occurring natural mycotoxins produced by several strains of *Aspergillus* fungi including *Aspergillus flavus, A. parasiticus, A. nomius, A. pseudotamarii, A. bombycis,* and *A. ochraceoroseus* (Cotty et al., 1994; Bhatnagar et al., 2003). These ubiquitous fungi occur in food, several crops and animal feeds before harvest or during storage leading to contamination of commodities by colonization, growth and toxin production. The fungi are known to produce aflatoxins as secondary metabolites under favorable conditions of temperature, moisture, and relative humidity (Trenk and Hartman, 1970; Bennett and Klich, 2003).

Aflatoxins are known to incur a wide range of adverse and toxic effects (both acute and chronic) on human and animal health upon ingestion, inhalation or even dermal or contact (Riley et.al., 1985; CAST, 2003). Among several kinds of aflatoxins including B_1, B_2, G_1, G_2 and M_1, aflatoxin B_1 (AFB1) is known as the most toxic, carcinogenic, and most prevalent

member of the group (IARC, 1993; Puschner, 2002; Richard, 2007). Several reports have shown significant impact on health of animals including pigs (Harvey et al., 1991), poultry birds (Miazzo et al., 2005), and turkey poults (Rauber et al., 2007) that were fed with elevated aflatoxin levels. Short term exposure to low aflatoxin levels through feeds may cause negligible physiological damage in animals, and long term ingestion of subchronic levels can significantly impair the performance of poultry birds (Jones et al., 1982), cause subchronic mycotoxicosis in mice (Casado et al., 2001) and rats (Theumer et al., 2009), may lead to impairment of drug-metabolizing enzymes in pig liver (Meissonnier et al., 2007), and can even result in residual aflatoxin levels in eggs (Oliveira et al., 2003). In addition to these adverse health effects, the huge economic losses due to recall of aflatoxin contaminated foods and feeds and the associated regulatory losses of managing the recalled products has led to widespread screening of foods and feeds for potential aflatoxin contamination. According to the Food and Agricultural Organization (FAO) mycotoxins affect 25% of the world's commodities each year (Mannon and Johnson, 1985). In the United States alone, the mean economic annual cost from crop losses due to mycotoxins is estimated to be 932 million USD (CAST, 2003).

Due to frequent occurrence and severe toxicity, several countries have set maximum permissible limits for aflatoxins in commodities of food and feeds. The US Food & Drug Administration established action levels as low as 20 ppb for feedstuffs and 0.5 ppb for aflatoxin M1 (Figure 1) in the United States. Because of the low permissible limits for aflatoxins and the associated high toxicity of aflatoxins impacting health even at subchronic exposure, the analytical methods for determination of aflatoxins need to be both specific and sensitive enough to quantify the trace levels. Conventional analytical methods such as thin layer chromatography (TLC), gas liquid chromatography (GLC), high pressure liquid chromatography (HPLC), need extensive preparation and sample cleanup to provide rapid and sensitive measurements of aflatoxins found in complex food and feed matrices. Food and feed industry is in constant pursuit of rapid methods

that may also be highly effective to assure the safety and security of foods, preventing accidental or intentional contamination and toxin outbreaks that may occur.

Immunoassay methods are considered to be rapid and reliable to use in routine diagnostic applications for aflatoxin detection. The general performance of immunoassays depend mainly on the use of molecular label markers such as fluorescent compounds, enzymes, radio isotopes or other signal markers, the format of the assays with competitive or indirect antigen detection, and the type and sensitivity of devices used for signal measurement. The majority of immunoassays using horseradish peroxidase (HRP) and alkaline phosphatase (AP) enzyme labels are employed in the enzyme-linked immunosorbent assays (ELISA) used in monitoring agricultural commodities for aflatoxins.

The immuno-PCR (iPCR) approach for sensitive antigen detection combines the advantages of immunoassays with the enormous DNA amplification potential of PCR. Often times this method involves use of highly specific primary antibodies for capture of antigen sandwiched with secondary DNA conjugated to antibodies specific for the antigen (Sano et al., 1992; Niemeyer and Blohm, 1996; Adler et al., 2003; Niemeyer et al., 2007). Using DNA as the amplifiable marker, iPCR typically leads to a 10 to 1,000 fold increase in the limit of detection over ELISA methods (Sano et al., 1992; Niemeyer et al., 2005) allowing the quantification of an antigen with greater rapidity and sensitivity. Since its introduction by Sano et al., in 1992, several immno-PCR approaches have been developed for detection of various high molecular weight antigens.

Aflatoxin detection has largely relied on competitive ELISA formats that are semi-quantitative and depend on the use of conjugated toxin molecules. However, the limitation of conventional immunoassay formats is largely due to the requirement that at least two epitopes of an antigen to be recognized and occupied (Lim et al., 2007). It is more challenging to develop a

sandwich immunoassay assay for low molecular weight hapten molecules such as aflatoxins since the antibodies are much larger in size (150 K Da) than the aflatoxin molecules (~0.312 K Da) themselves preventing combinatorial association of antibodies due to steric hindrance and limiting access of the antigen to secondary antibodies. It is also reported that the sandwich immunoassay for molecules of 1000 Daltons MW is less amenable for such applications (Lim et al., 2007; Quinton et al., 2010). However, sandwich immunoassays for homovanillic acid (MW 182.17; Quinton et al., 2010) and open sandwich ELISA for angiotensin II (MW 1046.18; Lim et al., 2007) have been conducted to prove that low molecular weight molecules can be used successfully in sandwich immunoassays. Though novel approaches such as the open sandwich assay for small molecules have been suggested for years (Ueda et al., 1996; Wei et al., 2006; Sakata et al., 2009; Shirasu et al., 2009), they may not be simple to carry out. Some researchers have attempted similar assays such as the solid phase immobilized epitope immunoassay (Grassi et al., 1996) for molecules of 1000-to-500 Dalton MW. Due to these inherent reasons, the use of immuno-PCR has been widely successful for quantifying high molecular weight proteins. However, it is unclear about how small a hapten may be to allow simultaneous recognition by two different antibodies.

The use of sensitive real-time immuno-PCR has not been exploited to quantitatively determine contamination of mycotoxins such as aflatoxin B1. In this study, we successfully demonstrated the use of noncompetitive sandwich immunoassay for aflatoxin B1 optimizing the assay with suitable monoclonal antibodies for their high specificity, rapid magnetic bead recovery, and sensitive detection and signal amplification potential of real-time PCR. The methodology developed here is a simplified noncompetitive sandwich immuno quantitative PCR (RT-iqPCR) approach for detection and quantification of aflatoxin B1. It can be conveniently used with minimal sample processing after organic solvent liquid extraction of complex food and mixed feeds matrices. By capturing aflatoxin with monoclonal antibodies and detecting with

reporter DNA conjugated polyclonal antibodies on protein G magnetic bead surface, we could quantify aflatoxin as low as 0.1 ppb. This assay can be used to eliminate false positives commonly encountered in an immunoassay and to quantify sub-chronic toxin levels.

Materials and methods

Afltoxin B1 and the antibodies used in the real-time immono-PCR assay.

Aflatoxin B1 standard used in this study was purchased from Supelco Analytical (Bellefonte, PA). It was supplied as an analytical standard containing 20 μg AFB1/ml (20, 000 ppb) in 100% methanol which was used to prepare known concentrations of aflatoxin by diluting in 60% ice-cold methanol prior to use in an immunoassay. Anti-aflatoxin B1 polyclonal antibody raised in rabbit (part #A-8679) and monoclonal anti-aflatoxin B1 (IgG1 isotype) produced in mouse (part # A-9555) were purchased from Sigma-Aldrich (St. Louis, MO). Three other monoclonal antibodies AFC-6 (part# sc-69861), AFC-7 (part# sc-69862) and AFC-13 (part# sc-69863) raised in mouse were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Other secondary antibodies (not toxin specific) of monoclonal mouse anti-rabbit IgG (part # 211-002-171); Rabbit anti-mouse IgG (part # 315-005-046) and biotinylated rabbit anti-mouse IgG (part # 315-065-003) along with biotin-binding streptavidin (part # 016-000-084) were purchased from Jackson ImmunoResearch Laboratories Inc., (West Grove, PA).

Magnetic beads.

To obtain a suitable solid support for better assay performance, we tested four different types of magnetic beads for immobilizing the antibodies to capture aflatoxin. M-270 carboxylic acid (part # 143.05D), M-280 tosylactivated (part# 142.03) and Dynabeads® protein G (part # 100-03D) were purchased from Invitrogen Dynal Biotech (Oslo, Norway). PureProteome™ Protein G magnetic beads (part # LSKMAGG02) were purchased from Millipore (Billerica, MA). The coating procedure used with the capture antibodies for the M-270, M-280 dynabeads and

protein G Dynabeads® was done as per the manufacturer's instructions and precipitation of the capture antibodies using PureProteome protein G magnetic beads was done in citric acid buffer (pH 5.0), due to effective immobilization of tested monoclonal antibodies at this pH. Immunomagnetic dynabeads were prepared using a semi-automatic bead retriever (part # 159-50) supplied by Dynal Biotech (Oslo, Norway).

Preparation of reporter DNA marker.

Signal generating detection antibodies in the immuno-PCR were prepared by conjugating the antibodies with reporter DNA. The reporter DNA marker was generated by amplifying a 563-bp fragment of firefly (*Photinus pyralis*) *luciferase* from the pGL2 plasmid vector (Catalog# E1641, Promega, Madison, WI). Preparation and PCR amplification of this fragment was done as described by (Wu et al., 2001). Briefly, the 563-bp portion of the *luciferase* gene was amplified using a 5'-C_6 amino-modified forward primer (5'-NH_2-(C_6)-GTTCGTCACATCTCATCTAC-3') so that the reporter DNA could contain a 5'-amino group that can be further used to link the DNA to antibody. PCR amplification was done using 0.2 μg of the pGL2 plasmid DNA in 50 μl of PCR reaction mix containing 0.5 M each of the pGL2A forward primer and the unmodified pGL2A reverse primer (5'-TCGGGTGTAATCAGAATAGC-3') synthesized by Integrated DNA Technologies (Coralville, IA), 0.2 mM dNTPs, and 0.08 units of GoTaq flexi DNA polymerase (Catalog # M829A, Promega, Madison, WI). The PCR conditions were as follows: hold at 95^{o}C for 5 min; 30 cycles of 94^{o}C for 45 sec, 53^{o}C for 30 sec, and 72^{o}C for 1 min. An extension step was included in the final cycle for 10 min at 72^{o}C. The PCR fragment was ethanol purified and quantified using nanodrop UV spectrophotometer ND-1000 (Nanodrop, Wilmongton, DE).

Conjugation of Reporter DNA to detection antibodies:

Conjugation of reporter DNA to detection antibodies was done in a two-step reaction scheme of experiments and carried out simultaneously as follows. A heterobifunctional crosslinker Sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC, catalog # 22622) was purchased from Thermo Fisher Scientific Inc. (Rockford, IL) which was supplied in no-weigh format microtubes of 2 mg. The purified 563-bp reporter DNA was linked with this Sulfo-SMCC as per the manufacturer's instructions. Briefly, 2 mg of Sulfo-SMCC was dissolved in 200 µl of dimethylformamide (DMF) just before use and a 10 µg stock was prepared in water. A 50 fold molar excess of this Sulfo-SMCC preparation was added to 83 picomoles of reporter DNA in water and incubated at room temperature for 30 minutes. This allowed the covalent conjugation of the amine-containing DNA with the *N*-hydroxysuccinimide (NHS) ester by forming amide bonds. Excess crosslinker was removed using G-25 sephadex column and the desalted Sulfo-SMCC linked reported DNA was immediately used for conjugation experiments with detection antibodies. To identify a suitable detection antibody for different assay formats, polyclonal detection antibodies of anti-aflatoxin, anti-mouse and anti-rabbit antibodies were prepared separately for conjugation with Sulfo-SMCC linked DNA.

Conjugation of Sulfo-SMCC linked DNA to antibodies can be done by chemically reducing the disulfide bonds in IgG molecules (Joerger et al., 1995). We used an immobilized TCEP (Tris [2-carboxyethyl] phosphine hydrochloride) disulfide reducing gel (part # 77712) supplied by Thermo Fisher Scientific Inc., (Rockford, IL). The TCEP gel does not show rapid oxidation seen with other reducing agents such as dithiotreitol (DTT) and β-mercaptoethanol (BME) (Han and Han, 1994). It was used because the recovery of the reduced antibodies could easily be centrifuged without the need for gel filtration which may lead to loss of sample and also it does not interfere with common sulfhydryl-reactive reagents such as maleimide crosslinkers (Haugland and Bhalgat, 1998). The reduction of the disulfide bonds in the antibodies was done

using the immobilized TCEP disulfide reducing gel as per the manufacturer's instructions. Briefly, in a microcentrifuge tube, 15 µl of the prewashed TCEP gel was mixed with 25 picomoles of detection antibody and 10mM EDTA (Ethylenediaminetetraacetic acid) added to prevent oxidation of generated sulfhydryl groups. The reduction reaction was performed in ultrapure water for 15 minutes under gentle shaking and the tube was centrifuged at 1000 x g to obtain the supernatant containing the reduced antibodies. The sulfhydryl-containing detector antibodies were immediately mixed with 95 µl of the desalted Sulfo-SMCC linked reporter DNA. The conjugation reaction mixture was incubated at room temperature (in the dark) for 30 minutes to encourage formation of covalent linkage between the malemide group of the SMCC molecules and the sulfhydryl groups of the TCEP reduced antibody molecules. Unbound molecules were further removed by buffer exchange using G-25 sephadex column and the antibody-DNA conjugate was used as the secondary detector antibody in further experiments. Other detection antibodies of anti-mouse and anti-rabbit antibodies (Jackson immunoResearch laboratories Inc., West Grove, PA) were also prepared similarly to test their general performance in immunoassay formats (explained later).

Another approach of conjugating detection antibody with DNA marker was also tested using biotinylated rabbit anti-mouse IgG molecules and biotin-binding streptavidin (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) as described by Zhou et al., 1993). As suggested by Niemeyer et al., (2005), pre conjugated streptavidin-DNA complex was utilized to avoid extensive incubation and washing steps. The DNA to be conjugated with streptavidin was generated using same primers mentioned above except that the forward primer was 5'-biotinylated instead of the 5'-amino group.

Sandwich immunoassay formats and immuno magnetic bead recovery of aflatoxin:

Due to lack of a standardized protocol for aflatoxin detection using real-time immuno-PCR, several experiments utilizing different immunoassay formats were conducted involving anti-aflatoxin antibodies to determine which one (s) showed the least nonspecific binding to DNA conjugated detection antibodies. We opted to use PureProteome^{TM} protein G magnetic beads as solid supports among the four bead types examined. The protocol that suitably worked for developing a standard curve using real-time PCR among all the assays conducted is further outlined here. A standard working stock of 80 ppb aflatoxin B1 was prepared in ice-cold 60:40 Methanol:Water solvent using the aflatoxin B1 standard supplied as a 20 μg/ml analytical standard in 100% methanol (Supelco Analytical, Bellefonte, PA). Different concentrations of aflatoxin B1 were prepared from this stock in separate 1.5 ml microcentrifuge tubes (100 μl of each standard) and were added with 1 μg (5 ul) of AFC-13 monoclonal anti-AFB1 antibodies. Toxin capture was performed at room temperature by gently shaking the tubes for 5 minutes. A 10 μl suspension of PureProteome^{TM} protein G magnetic beads prewashed and re-suspended in citrate-Phosphate buffer (pH 5.0) containing 4.7 g/L citric acid and 9.2 g/L dibasic sodium phosphate (Na_2HPO_4) dehydrate was added and incubated at room temperature with gentle shaking and carefully avoiding the settling of the beads. After a 15 min incubation, the magnetic beads were allowed to form a pellet using a magnet and the solvent containing unbound molecules was aspirated by pipette. The beads were further washed once with 200 μl of citrate-phosphate wash buffer and suspended in 25% normal rabbit serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) diluted in the same wash buffer to block the beads. After a 5 min blocking step, 100 μl of detection antibody (reporter DNA conjugated to polyclonal anti-AFB1 antibody at 0.0075 ng DNA/μl) diluted in wash buffer was added and incubated to allow for a 'sandwich' to take place. A short incubation time of 5 min was used to reduce nonspecific binding of the detection antibody. Immediate separation of the magnetic beads with mouse anti-

AFB1 monoclonal antibody-aflatoxin-rabbit anti-AFB1 polyclonal antibody sandwich was done by using magnetic retention and washed twice with PBS (pH 7.4) and twice again with sterile, molecular grade nuclease-free water (TekNova, part # W 3350). After the final wash step, the beads were transferred to nuclease-free 0.2 ml PCR tubes and heated for 10 min at 80^{o}C to release the bound molecules. After brief centrifugation at 5000 rpm, the supernatant was collected in new tubes and used for real-time PCR detection.

Triple antibody sandwich immunoassays were tested using two approaches. For the first approach, aflatoxin B1 (200 μl) was captured with 10 μl of undiluted anti-AFB1 mouse monoclonal antibody (AFC-6) before precipitating with protein G dynabeads (5 μl). A 200 μl of diluted A-8679 rabbit polyclonal anti-AFB1 antibodies (2 μg/ml PBS pH 7.4) was added as secondary IgGs and 100 μl of mouse monoclonal anti-rabbit IgGs tethered with reporter DNA were added as signal generating detection antibodies.

Several additional immunoassay formats were examined. One was a triple antibody sandwich immunoassay format that included the use of biotinylated anti-mouse antibodies (0.5 μg) as third IgG molecule that was labeled (after 3 PBS washing steps) by conjugating the biotin with 0.2 μg streptavidin-DNA complex. In this assay format, anti-AFB1 polyclonal antibodies were used to capture the aflatoxin before binding to the PureProteome protein G beads. AFC-6 mouse monoclonal antibodies (1:20) were then used as secondary antibodies to form a sandwich. Immuno-magnetic PureProteome protein G beads prepared by pre-conjugating with mouse monoclonal anti-rabbit IgGs were then used to precipitate the toxin bound polyclonal antibodies.

Real-time immuno quantitative PCR for detection and quantification of aflatoxin B_1.

The immunoassay in this paper is being developed as a model system that could easily be adapted for aflatoxin detection in a variety of food or animal feed samples using methanol:water as an extraction solvent. For the real-time PCR assay to be more sensitive, 2-fold dilutions of the reporter DNA were used and tested for the reaction efficiency. The development of a standard curve was done using 2 fold dilutions of aflatoxin working stocks prepared in a 60:40 methanol:water solvent and subjected to immunomagnetic bead based recovery of each standard using the sandwich immunoassay format mentioned above. The sensitivity of the assay results from amplifying the recovered DNA whereby the real time PCR signals were correlated to the initial toxin concentrations. The immuno-PCR assay was performed using an optimized assay using the MJ Research Opticon-2 real-time PCR detection system. Briefly, a 101-bp internal region of the reporter DNA was amplified in a 20 µl reaction mix containing 9.36 µl of template DNA and 1x concentration of PerfeCTa® SYBR® Green I FastMix® (Quanta BioSciences Inc., Gaithersburg, MD). Among three different primers pairs tested, an optimum primer combination of pGL2B primers was used at 80 nM concentration (pGL2B forward, 5'-GAACTGCCTGCGTCAGATTC-3'; pGL2B reverse 5'-AACCGTGATGGAATGGAACAAC-3'). The utility of ABsolute™ qPCR sybr® green mix (part # AB-1158, Thermo scientific, Rockford, IL) was also tested to compare with the PerfeCTa® fastmix®. Positive control of polyclonal anti-AFB1 antibody conjugated with DNA, antigen negative control that was processed same as the samples and a template negative control using water as template were included in each run. The optimized real-time PCR cycle parameters included a 95°C initial denaturation step for 30 sec, followed by 40 cycles of denaturation (95°C for 2 sec), and annealing (51°C for 25 sec). Fluorescence measurements were taken after each annealing step. A melting curve analysis to detect potential nonspecific products was done from 50°C to 90°C with signal acquisition at every 0.2°C melting rates for 1 sec hold time.

Calibration curve and quantitative real time-PCR analysis.

The increase in fluorescence signals after each PCR cycle during reporter DNA amplification was recorded automatically by the instrument. The cycle number where the fluorescence signal crosses a manually set threshold showing linear signal increase was labeled as the 'threshold cycle (C_t)'. The obtained C_t values were used to inversely correlate with antigen concentrations. The template negative controls or antigen negative controls would have the highest numerical C_t values. The C_t values were plotted against the logarithmic concentrations of the toxin standards for the calibration curve. Data analysis was done using a simple linear regression analysis of the C_t values against log concentrations and plotting of the amplification curves was done using SigmaPlot 11 software. A triplicate determination of each intra-assay calibration was performed and the average standard deviations (SDs) of C_t values were calculated.

Results and discussion

Development and optimization of immuno-PCR and assay formats for detection of Aflatoxin B_1.

Initial optimization and troubleshooting of real-time PCR was done to obtain higher signal amplification of reporter DNA conjugated onto detection antibodies and to insure little or no detectable fluorescence signal in negative controls without the formation of nonspecific products. Use of PerfeCTa® SYBR® Green I FastMix® over the ABsolute™ qPCR sybr® green mix showed better amplification efficiency and fast cycling in a gradient PCR setting with a primer concentration of 80 nM and showed no or little signal amplification with negative controls. Melting curve analyses were done in each iPCR reaction to insure the absence of nonspecific products. The reaction efficiency was tested to meet quantification requirements of real-time PCR using dilutions of antibody conjugated reporter DNA.

Covalent immobilization of anti-AFB1 antibodies and assay performance:

The use of different magnetic bead types and their assay performance as convenient solid supports for sandwich immunoassay was evaluated in direct and indirect noncompetitive aflatoxin detection along with screening of suitable pair of anti-aflatoxin monoclonal and polyclonal antibodies (Figure 2). Dynal magnetic beads offering different chemistries (M-270 carboxylic acid and Tosylactivated M-280 dynabeads) for covalent immobilization of IgG molecules were tested. M-270 carboxylic acid dynabeads processed as per the manufacturer's instructions showed higher nonspecific binding of our detection antibodies. M-280 Tosylactivated Dynabeads® performed comparatively better but required about 16-24 hours of conjugation time

for coating the beads with primary antibodies and may not ensure proper orientation of all primary antibodies essential for sandwich immunoassay. Though M-280 Tosylactivated Dynabeads® showed very little nonspecific binding when the DNA-conjugated secondary antibody reaction was limited to 15 min, we were not able to obtain a reliable standard curve for aflatoxin standards (Figures 3 and 4). Stringent washing steps with high salt Tris Buffer saline (TBS) with up to 1M NaCl, 1x phosphate buffered saline (PBS) from pH 7.4 to 8.0 and 0.1% to 0.2% Tween 20 could not eliminate nonspecific binding (data not shown). Poor toxin detection using the M-270 and M-280 Dynal beads may not only be due to the nature of the beads but may also be due to the chemistries involved in immobilization of anti-aflatoxin antibodies that may have an effect of antigen reactivity and capture of aflatoxin. Moreover, antibodies immobilized on these bead surfaces may either be in random array or in an oriented array with Fc or Fab portion bound onto bead surfaces. Such random immobilization of antibodies may denature the antibody itself irrespective of covalent or non-covalent adsorption onto the surface (Butler et al., 1992; Joshi et al., 1992; Butler et al., 1993). Loss of antigen specificity due to immobilization of antibodies may be due to steric hindrance by the solid support itself or by the adjacent antibodies. Though this phenomenon may vary with type of antibody, Butler et al., (1993) reported that among the several anti-fluorescyl capture antibodies tested, more than 90% of monoclonal and 75% of polyclonal antibodies were denatured by their passive adsorption. It has been suggested that reactivity of immobilized antibodies can be greatly improved by specifically adsorbing the capture antibodies onto suitable solid supports by the Fc region of the antibodies and that the Fab (antigen binding) portion be oriented away from the adsorbing surface of the bead (Lu et al., 1996).

Non-covalent immobilization of anti-AFB_1 antibodies and sandwich immunoassays.

We opted to use another approach of developing immunoassay formats using magnetic beads coated with protein G that usually offer faster (10-15 minute) immuno-precipitation of the IgG molecules. Protein G coated beads are most commonly used in non-covalent affinity purification of monoclonal and polyclonal antibodies isolated from various animal species as protein G is known to offer optimal orientation of antibodies by binding specifically to Fc fragments of IgG molecules (Grubb et al., 1982; Akerstrom et al., 1985). The protein G beads used in this study were found to bind IgG molecules efficiently even in diluted solvents such as methanol. We compared Dynal® and PureProteome® protein G magnetic beads.

Various options of blocking Dynal protein G magnetic beads to prevent nonspecific binding of secondary anti-AFB1 antibodies tethered with reporter DNA were attempted with little success. Moreover all three Dynal beads (not immobilized with captured antibodies) showed nonspecific binding of both the amino-modified reporter DNA with or without conjugated antibodies and the tosylactivated M-280 beads showed the least nonspecific binding of the labeled biomolecules (Figure 5). In order to do stringent washing to remove nonspecific binding, cross-linking the capture antibodies onto protein G dynabeads was also tested using Bis(sulfosuccinimidyl)suberate (BS3) cross-linking agent as per the manufacturer recommended protocol, but the sensitivity of the immunoassay reduced significantly without eliminating nonspecific binding (Figure 6). Dynal protein G beads, however showed less nonspecific binding and improved assay performance in terms of toxin detection when triple antibody sandwich assay was tested (Figure 7). Different mouse monoclonal antibodies (AFC-6, AFC-7 and AFC-13) were tested with Dynal protein G beads, and AFC-6 IgG1 showed improved assay performance (Figure 8).

PureProteome protein G beads were also used for triple antibody sandwich assay using rabbit polyclonal anti-AFB1 antibodies for toxin capture (Figure 9). Due to potential loss of antigen reactivity by the immobilized antibodies (Butler et al., 1992), introduction of a fourth anti-rabbit antibody molecule (already bound to protein G beads) to specifically immuno-precipitate the toxin captured rabbit antibodies worked well (Figure 2). The primary and secondary antibodies that worked better in this approach were the rabbit polyclonal anti-AFB1 IgGs (A-8679) and 1:20 diluted mouse monoclonal IgGs (AFC-6) respectively (Figure 10). Every step in the assay formats above were followed by three washings with 1x phosphate buffered saline (PBS) pH 7.4 containing 0.005% Tween 20. However, the approach of these multiple antibody sandwich assay formats involving DNA-conjugated or the biotinylated tertiary antibodies were rejected due to the requirement of too many wash steps and the high nonspecific signals in antigen negative controls that narrowed detection limits.

PureProteome protein G beads were found to be suitable solid supports for aflatoxin sandwich immunoassay involving two anti-aflatoxin antibodies. Toxin capture was evaluated using monoclonal or polyclonal anti-aflatoxin antibodies either by pre-binding onto magnetic beads or binding after capturing the toxin molecules (Figures 11 & 12). Improved assay performance was seen when mouse monoclonal anti-AFB1 antibodies and polyclonal anti-AFB1 antibodies were used in an indirect sandwich assay format of capturing the aflatoxin before immobilizing the antibodies onto PureProteome protein G beads. This indicated the suitability of these antibodies for the indirect assay format and immobilizing them onto beads prior to toxin capture can significantly reduce their reactivity leading to poor assay performance. After optimization and better assay performance of sensitivity, rapidity and specificity of aflatoxin detection, mouse monoclonal anti-aflatoxin antibodies were selected for toxin capture before binding onto magnetic beads. This was mainly done due to higher specificity of monoclonal antibodies and also significant loss of their reactivity if conjugated to use as secondary antibodies.

Indirect sandwich assay format was carried out on PureProteome protein G beads. Toxin capture was done using AFC-6 mouse monoclonal anti-AFB1 antibodies prior to immobilizing them on beads. Detection antibodies conjugated with DNA prepared using both antibodies were added at 1:10000 dilutions in PBS. Captured toxin standards were retrieved using magnetic bead based recovery and after washing to remove unwanted molecules, the beads were heated at 80^{o}C for 10 minutes to release template with reporter DNA before subjecting to optimized RT-iqPCR assay. Intra-assay comparison of the three replicates of each standard was done. A calibration curve using the diluted aflatoxin B1 standards prepared in 60:40 methanol:water solvent was generated and the cycle threshold (C_t) values were plotted in a linear regression analysis using SigmaPlot 11.0 software (Figures 13 & 14). Considering our objective of quantifying low aflatoxin concentrations less than 20 ppb, a narrow 2-fold dilution range of aflatoxin standards were prepared. The standard curve showed an acceptable real-time PCR efficiency of 99.52% with a slope of -3.33 indicating high sensitivity of the real-time PCR assay. The r^2 value of 0.9654 indicated that this curve can be confidently used to estimate aflatoxin concentrations of an unknown sample with corresponding C_t values. The differences in C_t values were very small due to the narrow dilution range of standards used. Thus, the real-time PCR assay was sensitive enough to differentiate the signals obtained for these standards. Though the assay performed with high sensitivity, higher signal amplification to clearly differentiate the antigen negative controls from the low level antigen standards was not observed. In order to increase the sensitivity of detecting lower antigen levels, evaluation of different monoclonal antibodies was done along with reduced stringency of washing steps to avoid loss of any specifically bound detection antibodies to the toxin in the sandwich immunoassay. By using other mouse monoclonal anti-AFB1 antibodies (AFC-13 mouse IgG1 isotype) as capture antibodies along with the DNA conjugated anti-AFB1 polyclonal antibody as detector antibodies, higher sensitivity was obtained in most simplified real time immuno quantitative -PCR without the use of stringent washing steps. Use of

AFC-13 anit-AFB1 antibodies thus provided higher sensitivity and a calibration curve using standards ranging from 0.1 to 10ppb was generated (Figure 15).

Nonspecific background from labeled antibodies and hook effect:

Nonspecific binding of detection antibodies was a major problem in most of the immuno-PCR assays we conducted. Use of highly specific antibodies with either DNA or biotin labeling showed nonspecific binding during the immunoassays contrary to the assumption that biotin labeling does not significantly affect the specificity or affinity of an antibody. Labeled antibodies show loss of affinity (Ghose et al., 1983; Pearson et al., 1998) and antigen specificity (Hoyer-Hansen et al., 2000). In agreement with the many immuno-PCR assays mentioned in literature, it was not possible to completely eliminate the nonspecific binding of the DNA conjugated antibodies onto bead surfaces. This could be mainly due to highly sensitive nature of the real-time immuno-PCR assay that can significantly amplify even the low rates of nonspecific binding. However, use of normal rabbit serum (diluted 1:2) as a blocking agent before addition of rabbit polyclonal detection antibodies was found to minimize the nonspecific background sufficient to distinguish the aflatoxin standards. Further reduction in nonspecific binding was obtained by extended blocking time, but the rapidity of the assay may be compromised. Several fold diluted detection antibodies conjugated with DNA could possibly reduce nonspecific binding. However the upper detection limit of the assay would be limited to by a certain level of antigen concentration. Thus, the quantitative assay developed here is based on how early the cycle threshold of a positive sample can be detected in comparison with the antigen negative controls. For detection purposes, the assay should be able to distinguish at least two-fold antigen dilutions in terms of cycle threshold for the retrieved reporter DNA amplification.

Stringent washing steps using higher concentration of non ionic detergent Tween 20 or PBS at pH 8.0 seem to eliminate the specific binding more than the nonspecifically bound detection antibodies. This could be due to much stronger binding capacity of the DNA-tethered antibodies. Weaker specific aflatoxin- antibody interactions could be due to low molecular weight of the toxin or limited accessibility of the captured antigen molecules available for secondary antibody binding. Thus, gentle washings steps using PBS pH 7.4 buffer followed washing with water should be sufficient enough to obtain a successful sandwich formation.

Another problem that we noticed was an excess antigen dependent 'hook effect' commonly seen in immunoassays due to high analyte concentrations (Rodbard et al., 1978; Fernando and Wilson, 1992; Killeen et al., 1993) which significantly limited assay performance. The hook effect, or prozone (high-dose) effect, is known to cause false-negative assay results if not properly addressed in samples with unknown toxin concentrations. We noticed the hook effect irrespective of the immunoassay formats we tested and noticeably dependent on the order of reagent addition to the immunoassay tubes (Figure 16). The hook effect can be shifted to increase the tolerance of immuno assays by using increased concentrations of labeled antibodies (Khosravi, 1990). However, increased amounts of DNA labeled antibodies only increased nonspecific binding limiting the assay sensitivity. The inclusion of additional wash cycles to remove excess antigens before adding labeled antibodies did not improve assay performance. We found that capturing the aflatoxin with the DNA-labeled anti-AFB1 antibodies prior to mixing with magnetic bead immobilized primary antibodies showed reduction in the hook effect. But, this approach may show high nonspecific binding of the labeled antibodies to other protein molecules in complex solvent extracted matrix such as methanol extracted food or mixed feed samples. Thus the assays were limited to toxin capture with highly specific non conjugated monoclonal antibodies before immobilization onto bead surface. However, high analyte concentrations can still be quantified using our real time immuno quantitative-PCR (RT-iqPCR)

using sample dilution protocols as suggested by several researchers encountering the hook effect (Saryan et al., 1989; Butch, 2000; Tang and Standage, 2000). We recommend that the unknown samples suspected to contain aflatoxin levels higher than the upper detection limits should be tested undiluted and diluted. If the diluted samples show lower cycle threshold in real-time PCR assays than the undiluted samples, then the unknown sample may be exhibiting the hook effect. More than one dilution can be done to precisely quantify the toxin levels and a correcting step for the dilution used should be included. If this strategy calls for increased assay costs, cost effective method of sample pooling can be done to see if any of the pooled samples show false negative results due to the hook effect (Cole et al., 1993).

The real time immuno quantitative PCR approach developed in this study for quantifying aflatoxin B1 can be potentially adapted to assays that use liquid phase organic solvent extractions. This was demonstrated by quickly capturing the antigen from the methanol:water solvent prior by antibody immobilization and immuno-precipitation using protein G magnetic beads that were then blocked and treated with DNA labeled antibodies. A calibration curve was obtained by signal amplification using highly sensitive real time immuno-quantitative PCR. This method offers rapid recovery with sensitive detection and quantification of aflatoxin using highly specific antibodies. The RT-iqPCR developed here offers detection of prozone effect for elevated toxin levels avoiding false negative estimations and could efficiently detect aflatoxin B_1 as low as 0.078 ppb. The lower and upper limits of quantification from this assay were at 0.1 ppb and 10 ppb respectively.

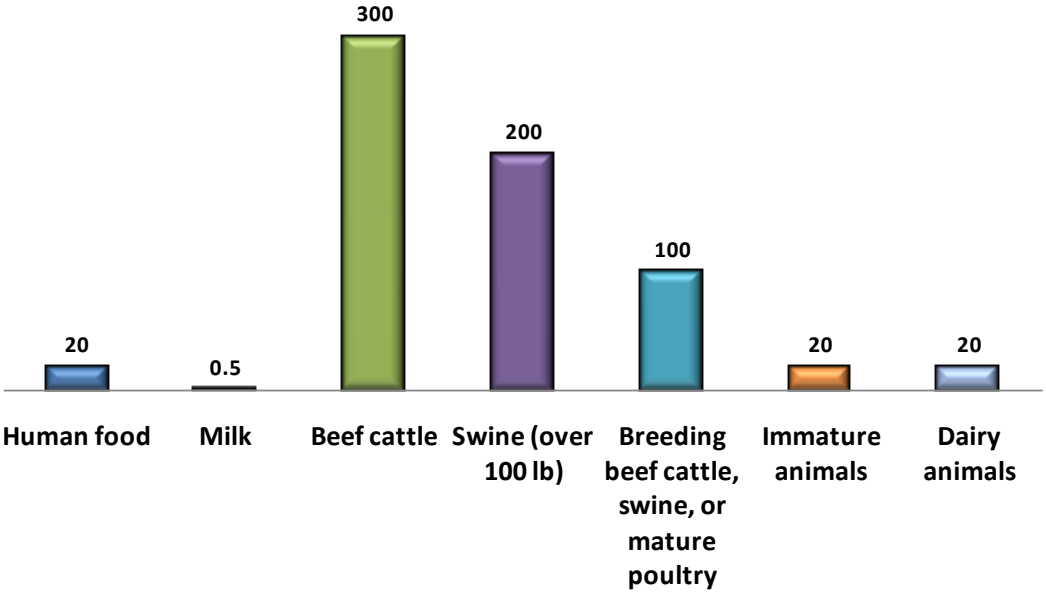


Figure 1. Action levels for aflatoxins in Animal Feeds, CPG 7126.33. US Food and Drug Administration, Rockville, MD, 1994 (*FDA, Section 683.100)* (http://www.fda.gov/ICECI/ComplianceManuals/CompliancePolicyGuidanceManual/ucm074703.htm).

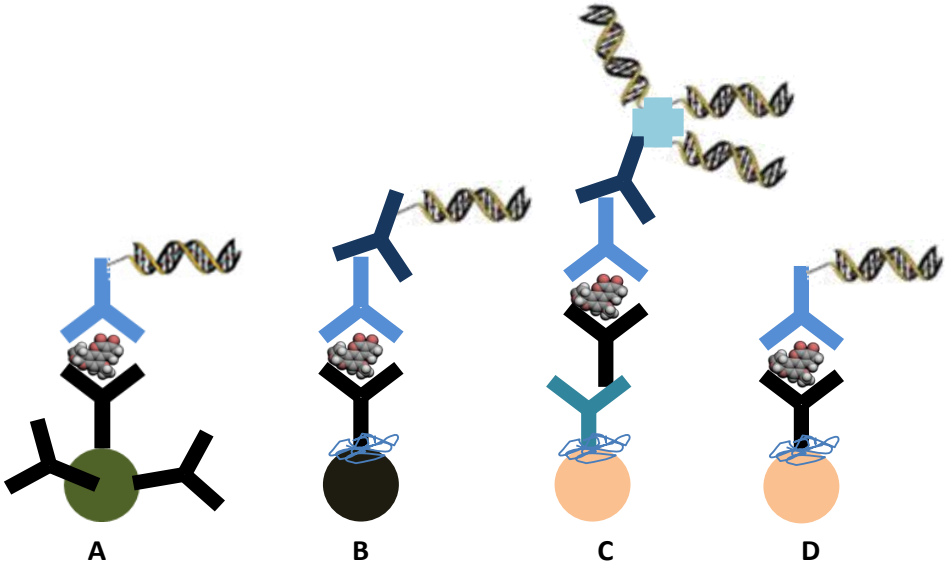


Figure 2. Illustration of selected Immunoassay formats used developing real time immuno quantitative PCR. **A.** Covalent immobilization of anti-AFB1 antibodies on dynabeads M270 or M280 dynabeads. **B.** Triple antibody sandwich assay with Dynal protein G beads. **C & D.** Four antibody sandwich format and two antibody sandwich assay format using PureProteome® protein G beads.

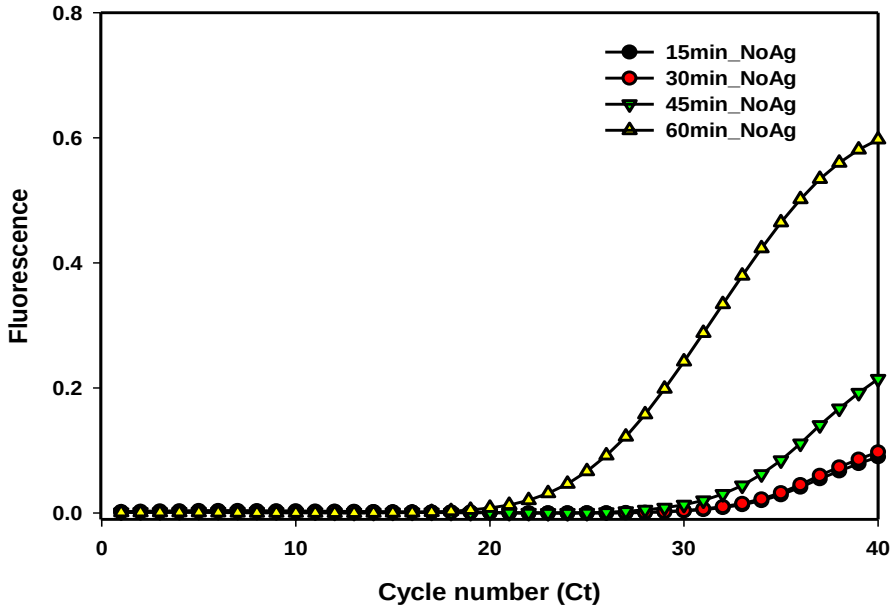


Figure 3. Incubation time dependent nonspecific binding of reporter DNA conjugated anti-aflatoxin polyclonal antibodies (pAb) onto M280 Tosylactivated Dynabeads® immobilized with pAb.

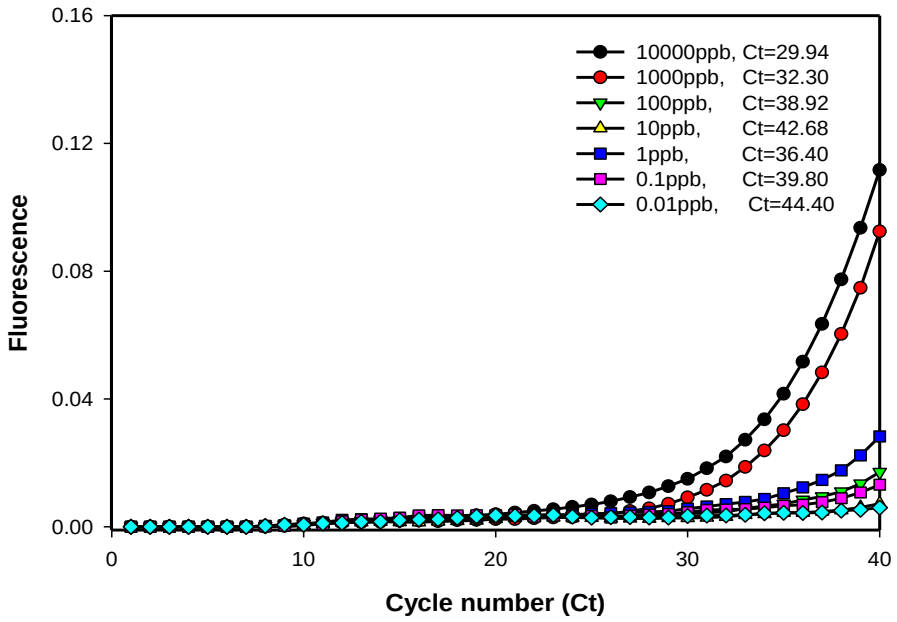


Figure 4.Immuno-PCR assay performance of M280 Tosylactivated Dynabeads® with nonlinear cycle threshold values for aflatoxin standards. The beads were conjugated with A-8679 anti-AFB1 polyclonal antibodies and toxin detection was done using AFC-6 mouse monoclonal antibodies conjugated with reporter DNA.

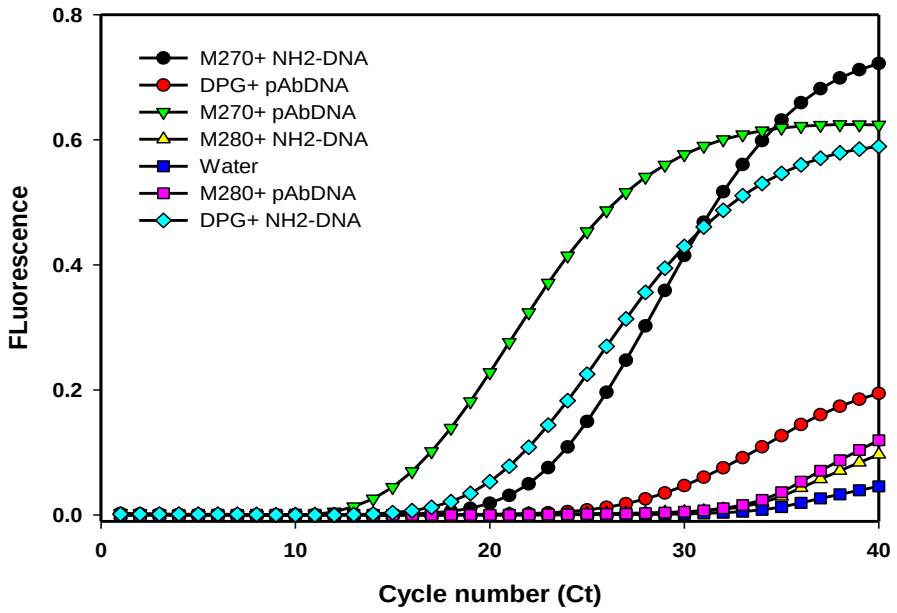


Figure 5. Comparison of magnetic beads showing nonspecific binding of amino-modified DNA (NH_2-DNA), and secondary anti-AFB1 polyclonal detection antibodies tethered with NH_2-DNA (pAbDNA).

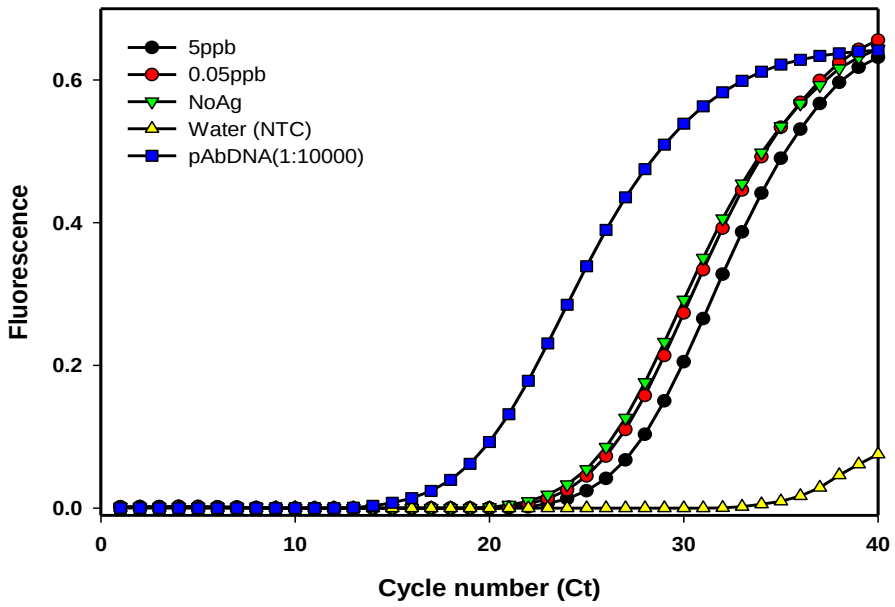


Figure 6. Increased nonspecific binding of anti-AFB_1 polyclonal antibodies tethered with reporter DNA (pAbDNA) onto protein G dynabeads cross-linked with pAb capture antibodies.

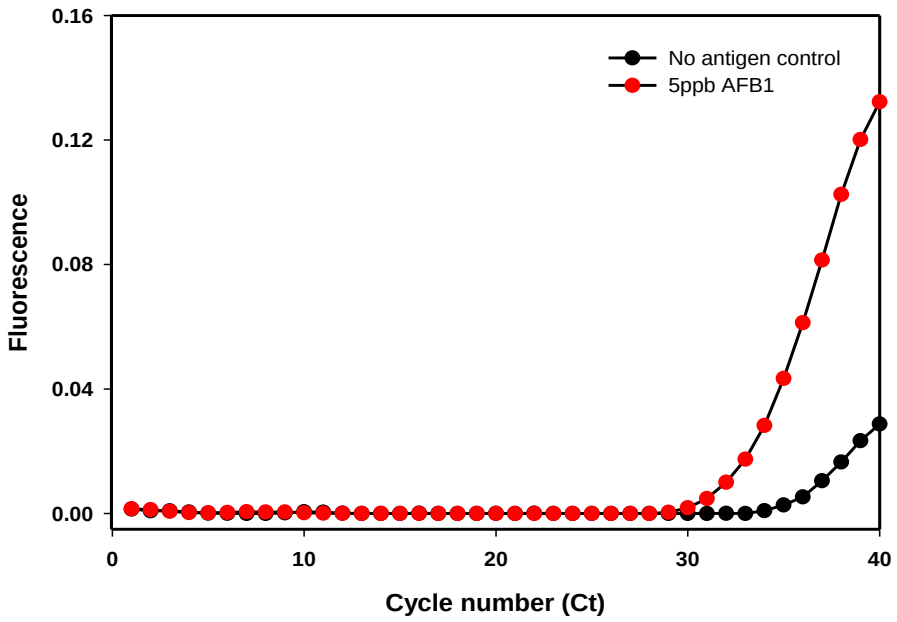


Figure 7. Triple antibody sandwich assay on protein G dynabeads performed using anti-AFB_1 mouse monoclonal antibodies (AFC-6) for toxin capture, anti-AFB_1 rabbit polyclonal secondary antibody (A-8679) and anti-rabbit mouse monoclonal IgG tethered with reporter DNA as the tertiary labeling antibody.

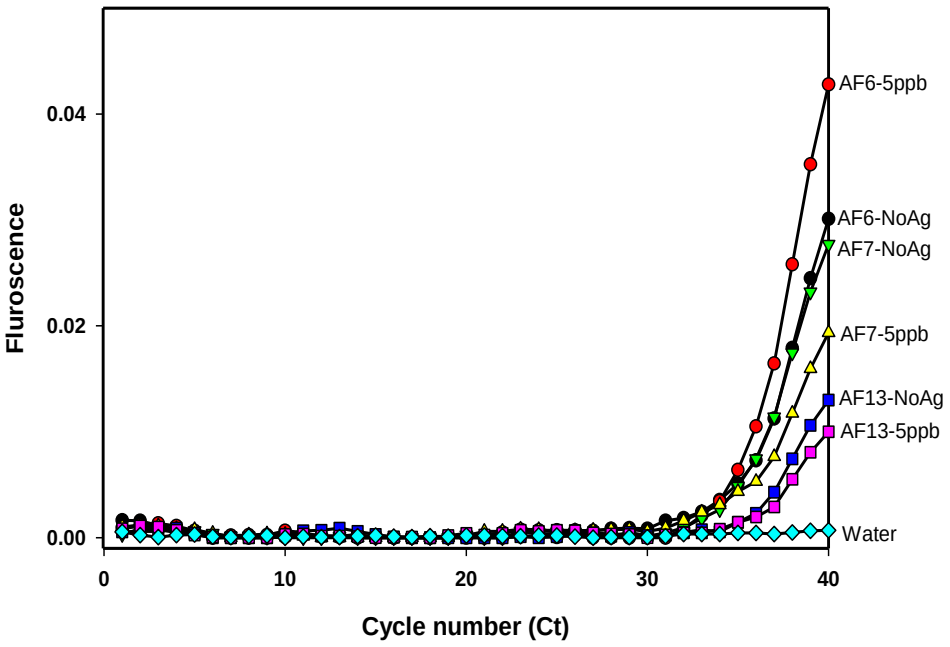


Figure 8. Performance of different anti-AFB_1 mouse monoclonal antibodies capturing aflatoxin in a triple antibody sandwich assay done using protein G dynabeads.

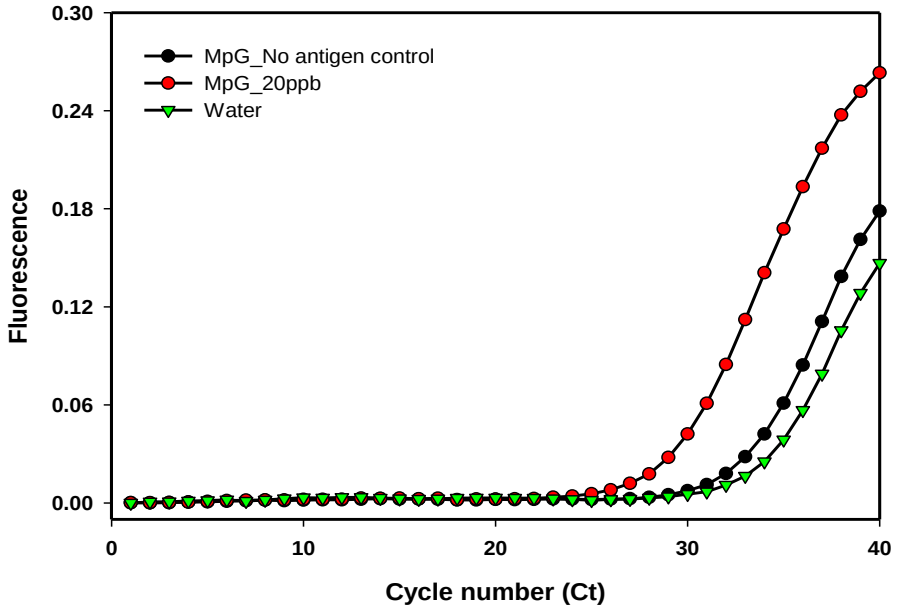


Figure 9. Use of biotinylated antibody streptavidin (STV) system on PureProteome protein G beads. A-8679 polyclonal antibodies (pAb) were used for toxin capture after immobilizing onto beads. Secondary antibodies were AFC-6 mouse monoclonal antibodies (1:20) and signal detection was done by labeling the third biotinylated anti-mouse monoclonal antibodies with STV-DNA mix.

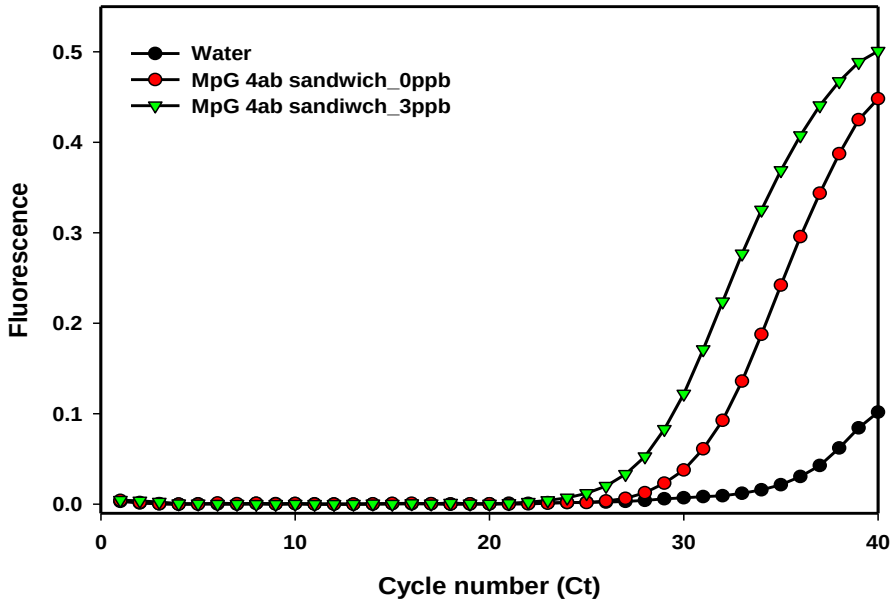


Figure 10. Use of biotinylated antibody-streptavidin (STV) system on PureProteome protein G beads immobilized with anti-rabbit antibodies (ARIgG). A-8679 polyclonal antibodies (pAb) were used for toxin capture and immuno-precipitated by the ARIgG bound beads. AFC-6 mouse monoclonal antibodies (1:20) were used as secondary antibodies and signal detection was done by labeling the tertiary biotinylated anti-mouse monoclonal antibodies with STV-DNA mix.

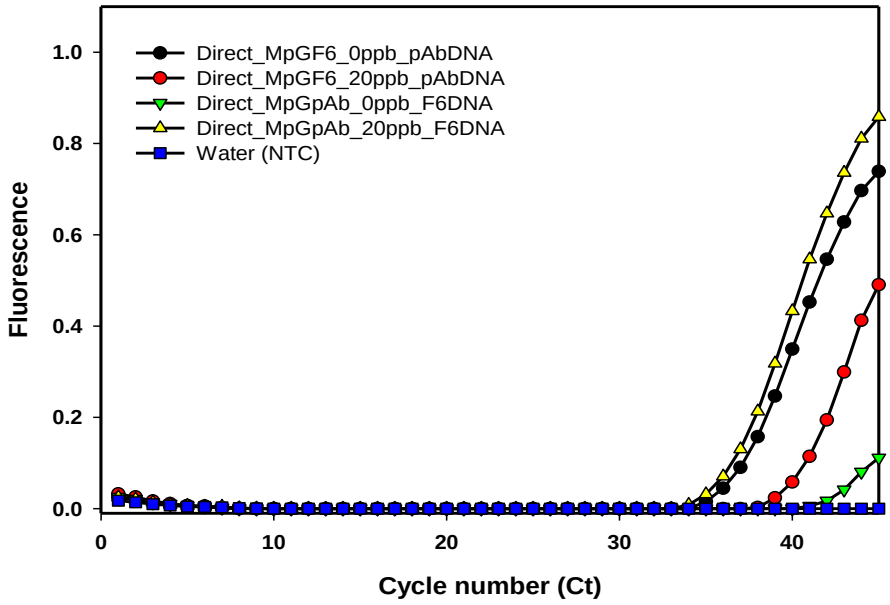


Figure 11. Double antibody sandwich assay format-1 on solid supports of PureProteome protein G beads. Toxin capture was done using either AFC-6 mouse monoclonal anti-AFB1 antibodies or the polyclonal anti-AFB1 (A-8679) antibodies after immobilizing onto beads (designated as MpGF6 or MpGpAb respectively). Both antibodies tethered with reporter DNA were also used as detection antibodies

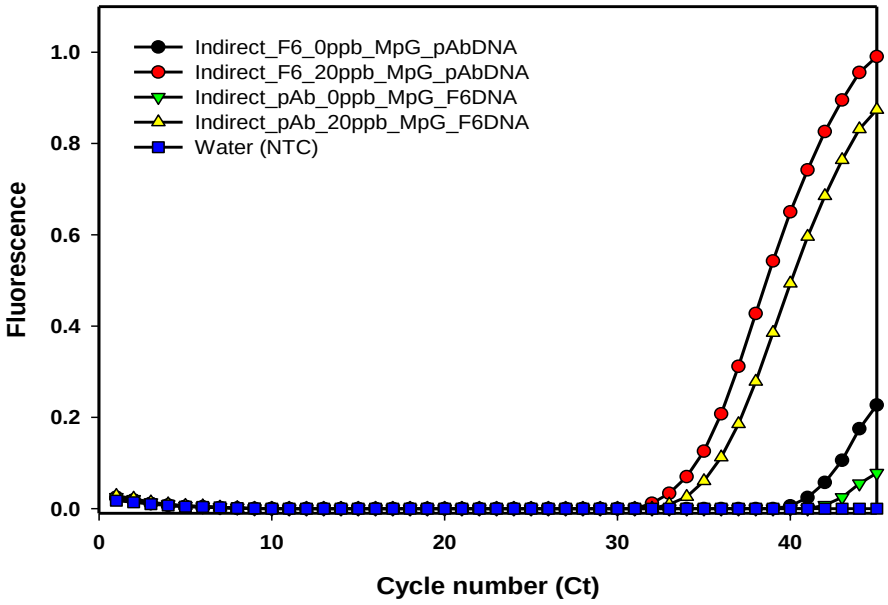


Figure 12. Double antibody sandwich assay format-2 on solid supports of PureProteome protein G beads. Toxin capture was done with anti-AFB_1 mouse monoclonal antibodies (AFC-6) or the polyclonal anti-AFB1 (A-8679) antibodies before immobilizing on PureProteome protein G beads to compare the indirect immunoassay performance. Both antibodies tethered with reporter DNA were also used as detection antibodies.

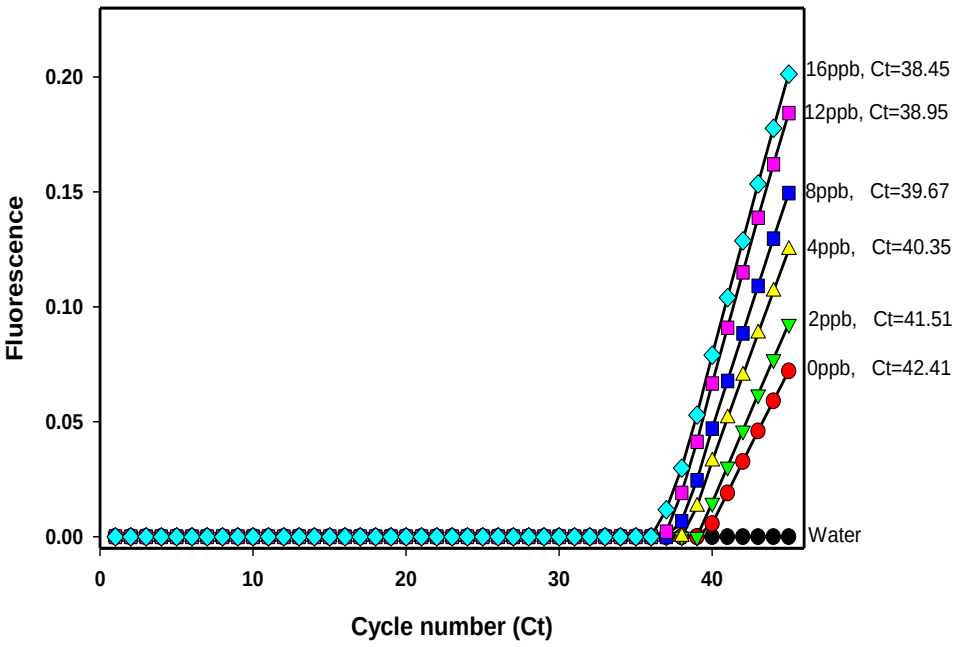


Figure 13. Calibration curve signals generated using AFC-6 anti-AFB_1 mouse monoclonal antibodies. Intra-assay comparison of three replicates of reporter DNA recovered from each standard was done.

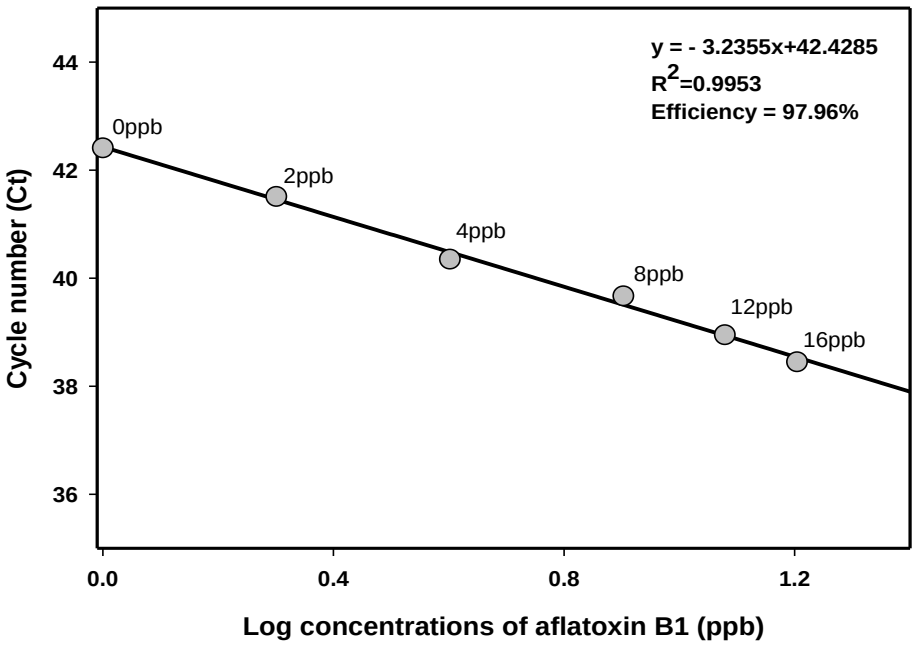


Figure 14. Calibration curve generated using AFC-6 anti-AFB_1 mouse monoclonal antibodies. Intra-assay comparison of three replicates of reporter DNA recovered from each standard was done.

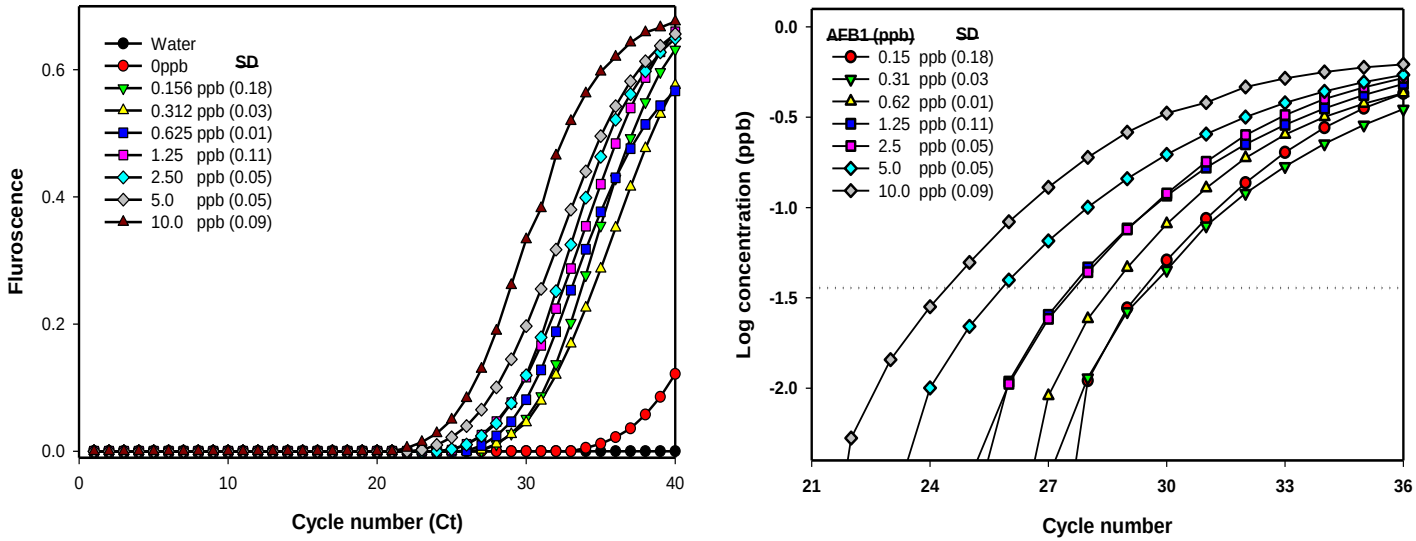


Figure 15. Calibration curve signals generated using AFC-13 anti-AFB_1 mouse monoclonal antibodies. Intra-assay comparison of three replicates of reporter DNA recovered from each standard was done.

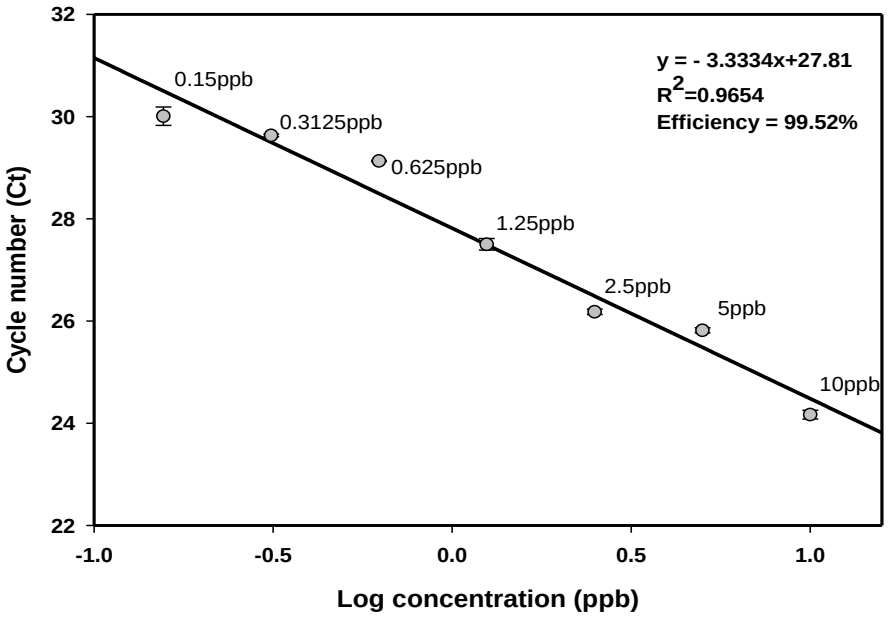


Figure 16. Calibration curve generated using AFC-13 anti-AFB_1 mouse monoclonal antibodies. Intra-assay comparison of three replicates of reporter DNA recovered from each standard was done.

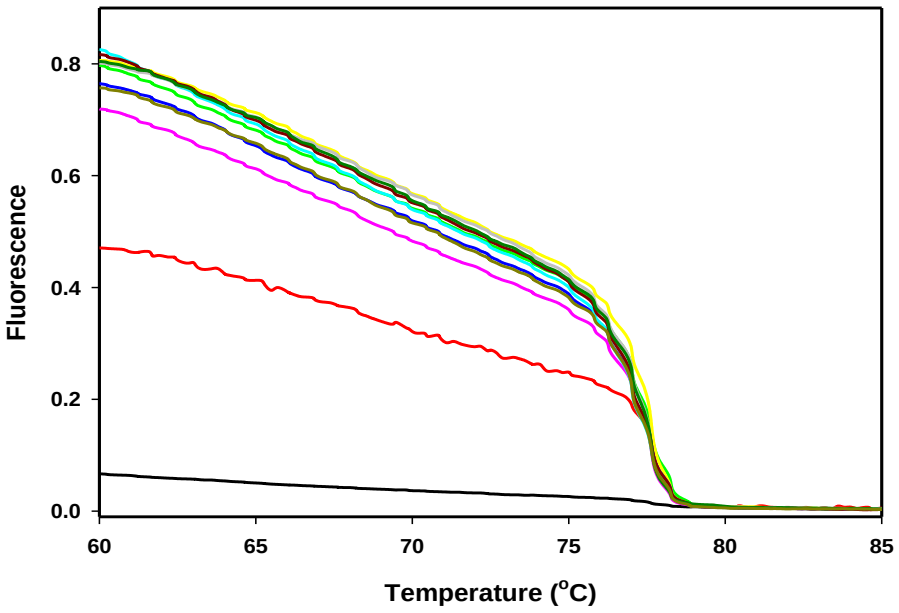


Figure 17. Melt curve analysis plots for aflatoxin B_1stadnards used for generating calibration curve where AFC-13 anti-AFB_1 mouse monoclonal antibodies were used for toxin capture.

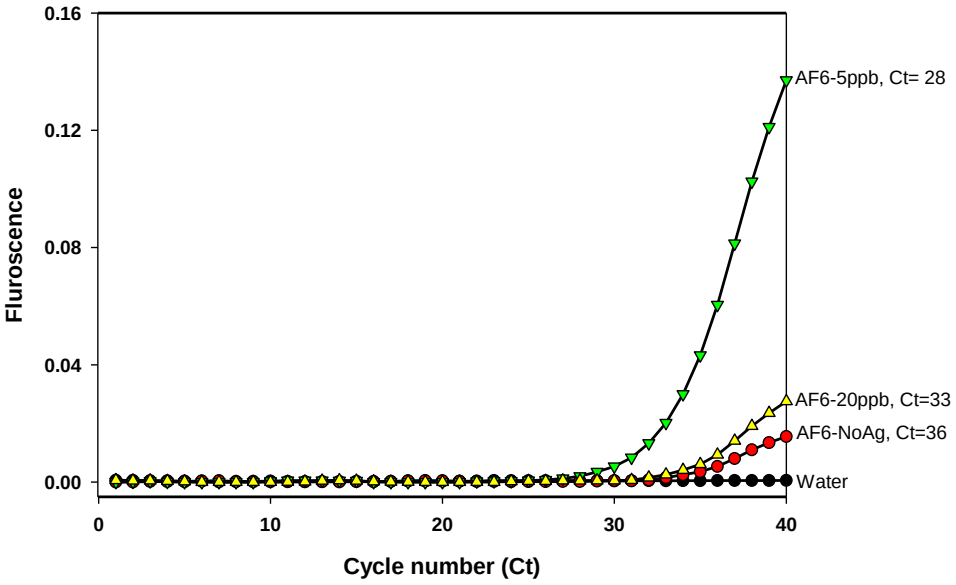


Figure 18. Detection of high dose hook effect at 20 ppb AFB_1concntration when AFC-6 anti-AFB_1 mouse monoclonal antibodies were used for capture in a triple antibody sandwich assay format on protein G dynabeads.

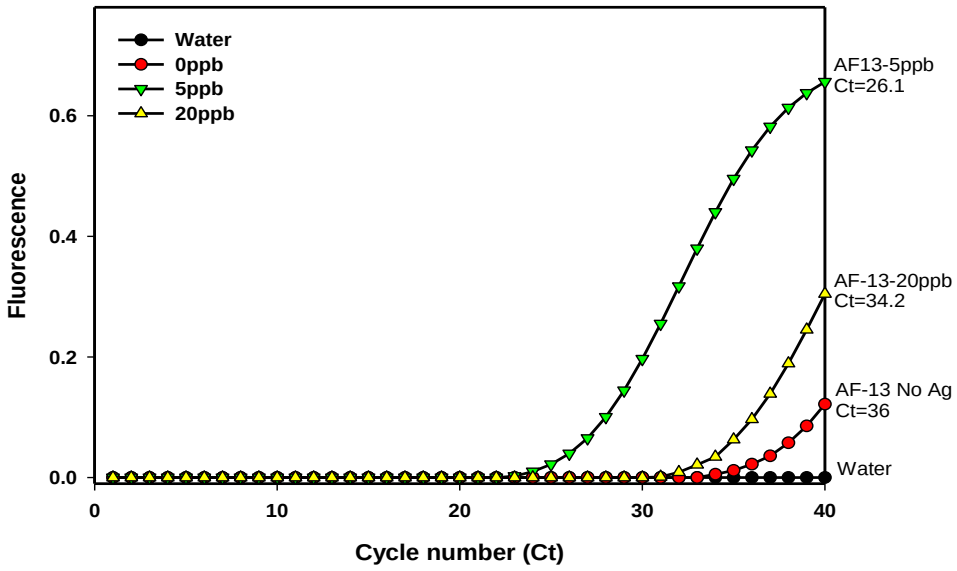


Figure 19. Detection of high dose hook effect at 20 ppb AFB_1concntration when AFC-13 anti-AFB_1 mouse monoclonal antibodies were used for capture in a triple antibody sandwich assay format on protein G dynabeads.

References

Adler, M., Wacker, R. and Niemeyer, C.M. (2003). A Real-Time immuno-PCR assay for routine ultrasensitive quantification of proteins. Biochem. Biophys. Res. Comm. 308, 240-250.

Akerstrom, B., Brodin, T., Reis, K. and Bjorck, L. (1985) Protein-G- A powerful tool for binding and detection of monoclonal and polyclonal antibodies. J. Immunol. 135, 2589-2592.

Bennett, J.W. and Klich, M. (2003) Mycotoxins. Clin Microbiol Rev 16, 497-516.

Bhatnagar, D., Ehrlich, K.C. and Cleveland, T.E. (2003). Molecular genetic analysis and regulation of aflatoxin biosynthesis. Appl. Microbiol. Biotechnol. 61, 83-93.

Butch, A.W. (2000) Dilution protocols for detection of hook effects/prozone phenomenon. Clinical Chemistry 46, 1719-1720.

Butler, J.E., Ni, L., Brown, W.R., Joshi, K.S., Chang, J., Rosenberg, B. and Voss, E.W., Jr. (1993) The immunochemistry of sandwich ELISA: Vi. Greater than 90 percent of monoclonal and 75 percent of polyclonal anti-fluorescyl capture antibodies (cAbs) are denatured by passive adsorption. Molecular Immunology 30, 1165-1175.

Butler, J.E., Ni, L., Nessler, R., Joshi, K.S., Suter, M., Rosenberg, B., Chang, J., Brown, W.R. and Cantarero, L.A. (1992) The physical and functional-behavior of capture antibodies adsorbed on polystyrene. J. Immunol. Methods 150, 77-90.

Casado, J.M., Theumer, M., Masih, D.T., Chulze, S. and Rubinstein, H.R. (2001) Experimental subchronic mycotoxicoses in mice: Individual and combined effects of dietary exposure to fumonisins and aflatoxin B_1. Food Chem. Toxicol. 39, 579-586.

CAST. (2003) Mycotoxins: Risks in plant, animal, and human systems. Task Force Report - Council for Agricultural Science and Technology, xvi + 199 pp.

Cole, T.G., Johnson, D., Eveland, B.J. and Nahm, M.H. (1993) Cost-effective method for detection of hook effect in tumor-marker immunometric assays. Clinical Chemistry 39, 695-696.

Cotty, P., Bayman, P., Egel, D. and Elias, K. (1994) Agriculture, aflatoxins and aspergillus. . In: R. Powell KA, A. and Peberdy, JF (Ed) The genus aspergillus: From taxonomy and genetics to industrial application. Plenum Press, New York, NY.

Fernando, S.A. and Wilson, G.S. (1992) Studies of the hook effect in the one-step sandwich immunoassay. J. Immunol. Methods 151, 47-66.

Ghose, T.I., Blair, A.H. and Kulkarni, P.N. (1983) Preparation of antibody-linked cyto-toxic agents. Methods in Enzymology 93, 280-333.

Grassi, J., Creminon, C., Frobert, Y., Etienne, E., Ezan, E., Volland, H. and Pradelles, P. (1996) Two different approaches for developing immunometric assays of haptens. Clinical Chemistry 42, 1532-1536.

Grubb, A., Grubb, R., Christensen, P. and Schalen, C. (1982) Isolation and some properties of an igg fc-binding protein from group-a streptococci type-15. International Archives of Allergy and Applied Immunology 67, 369-376.

Han, J.C. and Han, G.Y. (1994) A procedure for quantitative-determination of tris (2-carboxyethyl) phosphine, an odorless reducing agent more stable and effective than dithiothreitol. Anal. Biochem. 220, 5-10.

Harvey, R.B., Kubena, L.F., Elissalde, M.H., Corrier, D.E., Huff, W.E., Rottinghaus, G.E. and Clement, B.A. (1991) Cocontamination of swine diets by aflatoxin and diacetoxyscirpenol. J Vet Diagn Invest 3, 155-60.

Haugland, R.P. and Bhalgat, M.K. (1998) Preparation of avidin conjugates. Methods in Molecular Biology; Immunochemical protocols, Second edition, 185-196.

Hoyer-Hansen, G., Hamers, M., Pedersen, A.N., Nielsen, H.J., Brunner, N., Dano, K. and Stephens, R.W. (2000) Loss of ELISA specificity due to biotinylation of monoclonal antibodies. J. Immunol. Methods 235, 91-99.

IARC. (1993) Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. IARC monographs on the evaluation of carcinogenic risks to humans. Vol. 56. International Agency for Research on Cancer (IARC) Working Group, World Health Organization, Lyon, France, p. 467-488.

Joerger, R.D., Truby, T.M., Hendrickson, E.R., Young, R.M. and Ebersole, R.C. (1995) Analyte detection with DNA-labeled antibodies and polymerase chain-reaction. Clinical Chemistry 41, 1371-1377.

Jones, F.T., Hagler, W.H. and Hamilton, P.B. (1982) Association of low-levels of aflatoxin in feed with productivity losses in commercial broiler operations. Poult Sci 61, 861-868.

Joshi, K.S., Hoffmann, L.G. and Butler, J.E. (1992) The immunochemistry of sandwich ELISAs .5. The capture antibody performance of polyclonal antibody-enriched fractions prepared by various methods. Molecular Immunology 29, 971-981.

Khosravi, M.J. (1990) Shifting the hook effect in one-step immunometric assays. Clinical Chemistry 36, 169-170.

Killeen, A.A., Ramey, M.L. and Dean, J.J. (1993) High-dose hook effect in an immunoluminometric thyrotropin assay - the open-faced sandwich artifact. Annals of Clinical Biochemistry 30, 413-414.

Lim, S.L., Ichinose, H., Shinoda, T. and Ueda, H. (2007) Noncompetitive detection of low molecular weight peptides by open sandwich immunoassay. Anal. Chem. 79, 6193-6200.

Lu, B., Smyth, M.R. and Okennedy, R. (1996) Oriented immobilization of antibodies and its applications in immunoassays and immunosensors. Analyst 121, R29-R32.

Mannon, J. and Johnson, E. (1985) Fungi down on the farm. New Scientist, 12-16.

Meissonnier, G.M., Laffitte, J., Loiseau, N., Benoit, E., Raymond, I., Pinton, P., Cossalter, A.M., Bertin, G., Oswald, I.P. and Galtier, P. (2007) Selective impairment of drug-metabolizing

enzymes in pig liver during subchronic dietary exposure to aflatoxin B_1. Food Chem. Toxicol. 45, 2145-2154.

Miazzo, R., Peralta, M.F., Magnoli, C., Salvano, M., Ferrero, S., Chiacchiera, S.M., Carvalho, E.C., Rosa, C.A. and Dalcero, A. (2005) Efficacy of sodium bentonite as a detoxifier of broiler feed contaminated with aflatoxin and fumonisin. Poult Sci 84, 1-8.

Niemeyer, C. and Blohm, D. (1996) Protein detection with PCR (immuno-PCR). Nachrichten Aus Chemie Technik Und Laboratorium 44, 481-482.

Niemeyer, C.M., Adler, M. and Wacker, R. (2005) Lmmuno-PCR: High sensitivity detection of proteins by nucleic acid amplification. Trends in Biotechnology 23, 208-216.

Niemeyer, C.M., Adler, M. and Wacker, R. (2007) Detecting antigens by quantitative immuno-PCR. Nature Protocols 2, 1918-1930.

Oliveira, C.A., Rosmaninho, J.F., Castro, A.L., Butkeraitis, P., Reis, T.A. and Correa, B. (2003) Aflatoxin residues in eggs of laying japanese quail after long-term administration of rations containing low levels of aflatoxin B_1. Food Addit Contam 20, 648-53.

Pearson, J.E., Kane, J.W., Petraki-Kallioti, I., Gill, A. and Vadgama, P. (1998) Surface plasmon resonance: A study of the effect of biotinylation on the selection of antibodies for use in immunoassays. J. Immunol. Methods 221, 87-94.

Puschner, B. (2002) Mycotoxins. Vet Clin North Am Small Anim Pract 32, 409-19.

Quinton, J., Charruault, L., Nevers, M.C., Volland, H., Dognon, J.P., Creminon, C. and Taran, F. (2010) Toward the limits of sandwich immunoassay of very low molecular weight molecules. Anal. Chem. 82, 2536-2540.

Rauber, R.H., Dilkin, P., Giacomini, L.Z., Araujo de Almeida, C.A. and Mallmann, C.A. (2007) Performance of turkey poults fed different doses of aflatoxins in the diet. Poult. Sci. 86, 1620-1624.

Richard, J.L. (2007) Some major mycotoxins and their mycotoxicoses-an overview. Int. J. Food Microbiol. 119, 3-10.

Riley, R.T., Kemppainen, B.W. and Norred, W.P. (1985) Penetration of aflatoxins through isolated human epidermis. J Toxicol Environ Health 15, 769-77.

Rodbard, D., Feldman, Y., Jaffe, M.L. and Miles, L.E.M. (1978) Kinetics of 2-site immunoradiometric (sandwich) assays .2. Studies on nature of high-dose hook effect. Immunochemistry 15, 77-82.

Sakata, T., Ihara, M., Makino, I., Miyahara, Y. and Ueda, H. (2009) Open sandwich-based immuno-transistor for label-free and noncompetitive detection of low molecular weight antigen. Anal Chem 81, 7532-7.

Sano, T., Smith, C.L. and Cantor, C.R. (1992) Immuno-PCR - very sensitive antigen-detection by means of specific antibody-DNA conjugates. Science 258, 120-122.

Saryan, J.A., Garrett, P.E. and Kurtz, S.R. (1989) Failure to detect extremely high-levels of serum ige with an immunoradiometric assay. Annals of Allergy 63, 322-324.

Shirasu, N., Onodera, T., Nagatomo, K., Shimohigashi, Y., Toko, K. and Matsumoto, K. (2009) Noncompetitive immunodetection of benzaldehyde by open sandwich ELISA. Analytical Sciences 25, 1095-1100.

Tang, M.E. and Standage, J.C. (2000) Dilution protocols for detection of hook effects/prozone phenomenon. Clinical Chemistry 46, 1720-1721.

Theumer, M.G., Canepa, M.C., Lopez, A.G., Mary, V.S., Dambolena, J.S. and Rubinstein, H.R. (2009) Subchronic mycotoxicoses in wistar rats: Assessment of the in vivo and in vitro genotoxicity induced by fumonisins and aflatoxin B_1, and oxidative stress biomarkers status. Toxicology 268, 104-110.

Trenk, H.L. and Hartman, P.A. (1970) Effects of moisture content and temperature on aflatoxin production in corn. Appl Microbiol 19, 781-4.

Ueda, H., Tsumoto, K., Kubota, K., Suzuki, E., Nagamune, T., Nishimura, H., Schueler, P.A., Winter, G., Kumagai, I. and Mahoney, W.C. (1996) Open sandwich ELISA: A novel immunoassay based on the interchain interaction of antibody variable region. Nature Biotechnology 14, 1714-1718.

Wei, O.D., Lee, M., Yu, X., Lee, E.K., Seong, G.H., Choo, J. and Cho, Y.W. (2006) Development of an open sandwich fluoroimmunoassay based on fluorescence resonance energy transfer. Anal. Biochem. 358, 31-37.

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

Zhou, H., Fisher, R.J. and Papas, T.S. (1993) Universal immuno-PCR for ultra-sensitive target protein-detection. Nucleic Acids Research 21, 6038-6039.

CHAPTER IV

SENSITIVE QUANTIFICATION OF AFLATOXIN CONTENT IN ANIMAL FEEDS AND FEED GRAINS USING REAL TIME-IMMUNO QUANTITATIVE PCR (RT-iqPCR) AND IMMUNOMAGNETIC BEAD BASED RECOVERY

Introduction

Safety and security of animal feeds is a global concern due to the proven deleterious effects on the health of animals fed with unsafe feeds. Concern of animal feed safety has risen during last few decades because of the transfer of certain hazards from unsafe feeds into the food chain impacting human health. In addition, these implications affect trade and management of unsafe animal feeds and associated monetary losses from the rejected commodities. The criterion for selecting hazards of importance in animal feed is mainly based on the relevance of hazard to public health, extent of occurrence of the hazard and the impact of the hazard on trade (FAO/WHO, 2008). The majority of these undesirable substances comprise chemical (e.g., dioxins, mycotoxins, heavy metals, drug residues, pesticides) and microbiological hazards (e.g., *Salmonella, Brucella*).

Mycotoxins (secondary metabolites of toxigenic molds) are one of the commonly encountered deleterious agents in animal feeds known to contaminate nearly 25% of the world's food crops annually (Mannon and Johnson, 1985; FAO, 2004). Mycotoxins and the infestation of toxigenic fungi occur before or after harvest of several food crops of grain, oilseeds, fruits and vegetables resulting in serious human and animal health consequences. In the United States, mycotoxin related losses occur mainly as loss of animal health affecting animal producers causing loss of monetary benefits to the feed industry due to rejected lots of unsafe feeds. The annual mycotoxin related losses in United States is estimated to be ranging from $0.5 million to over $1.5 billion (Vardon et al., 2003). The Council for Agricultural Science and Technology (CAST) estimated crop losses due to mycotoxin contamination of corn, wheat and peanuts to a mean economic annual cost of $932 million and an average cost of $466 million from the enforcement of regulations, and quality control measures (CAST, 2003). Other costs may include increased health and veterinary care costs, insurance costs and investments in research and quality control measures.

Mycotoxins are considered as unavoidable natural contaminants of foods and feedstuffs due to the lack of a single approach to eliminate these fungal toxins that are produced by several strains of ubiquitous fungi. The major genera of fungi producing mycotoxins are *Aspergillus, Fusarium* and *Penicilium.* The severity of fungal infestation and mycotoxin production is promoted when conditions of temperature, excessive moisture, relative humidity, drought, insect damage, and variation in crop harvesting practices are favorable for growth of molds (Trenk and Hartman, 1970; Bennett and Klich, 2003; Reddy et al., 2005; Abbas et al., 2007; Bircan et al., 2008). Among the nearly 400 mycotoxins that are known, the important toxins of concern include aflatoxins, fumonisins, ochratoxin A (OTA), deoxynivalenol (DON or vomitoxin), zearalenone, T-2 toxin and T-2 like toxins. The World Health Organization (WHO)-International Agency for Research on Cancer during 1993, evaluated the carcinogenic potential of several mycotoxins and

classified aflatoxins as carcinogenic to humans (Group 1), Ochratoxins and fumonisins as possible carcinogens (Group 2B) (IARC, 1993; Vainio et al., 1994).

Aflatoxins are commonly encountered in foodstuffs and animal feeds worldwide and are mainly produced by the *Aspergillus* group of fungi. Species of *Aspergillus* fungi producing aflatoxins include *Aspergillus flavus, A. parasiticus, A. nomius, A. pseudotamarii, A. bombycis,* and *A. ochraceoroseus* (Cotty et al., 1994; Bhatnagar et al., 2003) with *A. flavus* and *A. parasiticus* being the most common ones. There are several types of aflatoxins including B_1, B_2, G_1, G_2 and M_1. Aflatoxin B_1 (AFB1) is the most toxic and prevalent member of the group (IARC, 1993; Puschner, 2002; Richard, 2007).

Aflatoxins, and specifically the AFB_1 type, are known to result in a wide range of adverse acute and chronic toxic effects on human and animal health irrespective of the mode of entry whether via ingestion, inhalation, or dermal contact (Riley et al., 1985; CAST, 2003). In domestic animals such as swine, cattle and poultry mycotoxins are known to cause several toxic effects of stunted growth, low feed conversion and reproduction, immuno suppression, reduced vaccination efficacy, and damage to liver and other organs. Many studies report that the severity of aflatoxin toxicity is mainly dependent on dose, age, duration of feeding, and other factors such as stresses affecting an animal. The toxic effects of aflatoxins (aflatoxicosis) can be distinguished as acute and chronic toxicities based on the durations and dosage of exposure (Leeson et al., 1995). Several reports have documented the significant impact on the health of animals including pigs (Harvey et al., 1991), poultry birds (Miazzo et al., 2005), and turkey poults (Rauber et al., 2007) that have been fed aflatoxin containing feeds. There is no safe level of aflatoxin chronic exposure as even low mycotoxin level can result in reduced productivity and increased susceptibility to infectious diseases (Hussein and Brasel, 2001). The long term ingestion of aflatoxins even at below permissible levels can significantly impair the performance of poultry birds. (Jones et al., 1982), cause subchronic mycotoxicosis in mice (Casado et al., 2001) and in rats (Theumer et al.,

2009), may lead to impairment of drug-metabolizing enzymes in pig liver (Meissonnier et al., 2007), and can even result in residual aflatoxin levels in eggs (Oliveira et al., 2003).

In order to avoid ill effects on human and animal health due to frequent occurrence and associated toxicity of aflatoxins, several countries have set maximum permissible limits in commodities of food and feeds. These limits are not universal to all the countries. For example, in the United States, US Food & Drug Administration has set the action levels for aflatoxins to be 20 ppb for feedstuffs and 0.5 ppb for aflatoxin M_1 (*http://www.fda.gov/ICECI/ComplianceManuals/CompliancePolicyGuidanceManual/ucm074703.htm*) and in the European Union, the regulatory limits for aflatoxin B1 in foodstuffs is at 2 ppb and 0.05 ppb for aflatoxin M_1 (*http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:050:0008:0012:EN:PDF*).

Because of the low permissible limits for aflatoxins and the associated high toxicity of aflatoxins impacting health even at sub-chronic exposure, the analytical methods for determination of aflatoxins need to be both sensitive and specific to be able to quantify trace levels. Aiming to achieve safety and security of animal feeds and foodstuffs preventing threat of aflatoxin contamination and the associated regulatory/trade losses, the food and feed industry is in constant pursuit of rapid and reliable methods for detection and quantification of aflatoxins. Among several available methods for aflatoxin detection, immunoassay methods are proven to provide such assurance during routine diagnostic applications of aflatoxin detection.

Recently, immuno-PCR (iPCR) approaches that combine the advantages of immunoassays with enormous DNA amplification potential of PCR have become popular for sensitive antigen detection. Often times this method involves the use of highly specific primary antibodies for capture of antigens sandwiched with secondary antibodies specific for the antigen that are conjugated (tethered) with DNA (Sano et al., 1992; Niemeyer and Blohm, 1996; Adler et

al., 2003; Niemeyer et al., 2007). Boasting a 10-to-1,000 fold increase in limit of detection than the traditional ELISA methods (Sano et al., 1992; Niemeyer et al., 2005), immuno-PCR allows quantification of an antigen with greater rapidity and sensitivity.

However, the use of this highly sensitive real time immuno-PCR approach has not been exploited to quantitatively determine contamination of mycotoxins such as aflatoxin B1 in complex matrices of foodstuffs, animal feeds, and feed grains. In this study, we demonstrate the use of immuno-PCR assay for sensitive detection and quantification of chronic levels of aflatoxin B1 in animal feeds and feed grains. The methodology developed here is a simplified noncompetitive sandwich immuno quantitative PCR (RT-iqPCR) approach for detection and quantification of aflatoxin B1. It can be conveniently used without sample cleanup using the organic solvent liquid extraction phase from extracted complex food and mixed feeds matrices. With this approach we could quantify as low as 0.1 ppb of aflatoxin B1. This assay can also be used to eliminate false negative samples commonly encountered in immunoassays and to quantify sub-chronic aflatoxin levels. Some of the advantages in quantifying low levels of aflatoxins beyond the detection limits of popular ELISA methods are that one can establish a stricter quality assurance of finished products for better trade and export value, eliminate transfer of toxins in the food chain in commodities such as milk and eggs, and accurate proper diagnosis in the case of chronic toxicity to humans and animals.

Materials and Methods

Animal feeds and feed grain samples.

Finished feed samples were collected to determine if toxins were present. Four finished animal feed samples were obtained from the Willard Sparks beef Research Center, Animal Science Department Oklahoma State University. A five pound sample of each poultry feed, dairy feed type 1 and type 2, horse feed and a corn sample (used as feed ingredient) were collected directly from the storage facility. The animal feeds selected contained the most commonly used ingredients in the respective animal diets. The samples were properly mixed in a large container and ground to fine powder using an electric coffee grinder. A finely ground food sample of yellow corn meal was also obtained from a local grocery store for comparison.

Mycotoxin extraction.

The finely ground feed grain samples were subjected to methanol (HPLC grade) extraction as follows. A 50 gm ground sample was mixed with 100 ml of 60:40 methanol:water solvent in a screw cap tube and left for 30 min with vigorous shaking at room temperature. After brief centrifugation at 3000 rpm, supernatant was collected in a separate tube. The sample extractions were stored at 4^{o}C and subjected to magnetic bead recovery of aflatoxin on the same day as explained later.

Spiking of feed and food samples.

A 50 gm of finely ground sample was spiked by adding 10 ml of 200 ppb of aflatoxin B1 prepared in 60% methanol. After mixing vigorously, the spiked samples were stabilized at room temperature for 30 minutes. Extractions of spiked samples were done as described above using 90 ml of 60:40 methanol:water solvent to collect clear supernatant. The extracted samples were stored at 4^{o}C and subjected to magnetic bead recovery of aflatoxin on the same day as explained later.

Afltoxin B1 and anti-aflatoxin antibodies used in the real-time immono-PCR assay.

An analytical standard of Aflatoxin B1 was purchased from Supelco Analytical (Bellefonte, PA) supplied at 20 μg AFB1/ml (20,000 ppb) in 100% methanol. Anti-aflatoxin B1 polyclonal antibody raised in rabbit (part #A-8679) was supplied by Sigma-Aldrich (St. Louis, MO), and monoclonal anti-aflatoxin B1 (AFC-13 IgG1 isotype) produced in mouse (part# sc-69863) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Secondary antibody preparations for toxin detection were done by conjugating the above polyclonal anti-aflatoxin antibody with DNA as explained below.

Magnetic beads.

PureProteome^{TM} Protein G magnetic beads (part # LSKMAGG02) were purchased from Millipore (Billerica, MA) and used as a solid support for sandwich immunoassays. Precipitation of the capture antibodies using these beads was done in citric acid buffer at pH 5.0.

Preparation of reporter DNA marker.

Our signal generating complex utilized a reporter DNA oligonucleotides used as a label on secondary antibodies. The reporter DNA marker was generated by amplifying a 563- bp fragment of firefly (*Photinus pyralis*) *luciferase* from the pGL2 plasmid vector (Catalog# E1641, Promega, Madison, WI). Preparation and PCR amplification of this fragment was done as described previously (Wu et al., 2001). Briefly, the 563-bp portion of the luciferase gene was amplified using a 5'-C_6 amino-modified forward primer (5'-NH_2-(C_6)-GTTCGTCACATCTCATCTAC-3') so that the reporter DNA could contain a 5'-amino group that can be further used to link the DNA to antibody. PCR amplification was done using 0.2 μg of the pGL2 plasmid DNA in 50 μl of PCR reaction mix containing 0.5 M each of the pGL2A forward primer and the unmodified pGL2A reverse primer (5'-TCGGGTGTAATCAGAATAGC-3') synthesized by Integrated DNA Technologies (Coralville, IA), 0.2 mM dNTPs, and 0.08 units of GoTaq flexi DNA polymerase (Catalog # M829A, Promega, Madison, WI).The PCR conditions were as follows: hold at 95^{o}C for 5 min; 30 cycles of 94^{o}C for 45 sec, 53^{o}C for 30 sec, and 72^{o}C for 1 min. An extension step was included in the final cycle for 10 min at 72^{o}C. The PCR fragment was ethanol purified and quantified using Nanodrop UV spectrophotometer ND-1000 (Nanodrop, Wilmongton, DE).

Conjugation of Reporter DNA to anti-afaltoxin polyclonal antibody.

Chemical conjugation of reporter DNA to polyclonal antibodies was done using a heterobifunctional crosslinker, Sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC, catalog # 22622) purchased from Thermo Fisher Scientific Inc. (Rockford, IL). The reaction scheme consisted of experiments that were carried out simultaneously in two steps of generating a reporter DNA-Sulfo-SMCC complex and preparation of reduced antibodies for conjugation. First, the purified 563-bp reporter DNA was linked with

Sulfo-SMCC as per the manufacturer's instructions. Briefly, 2 mg of pre-weighed Sulfo-SMCC was dissolved in 200 µl of dimethylformamide (DMF) just before use to prepare 10 µg stock in water. A 50-fold molar excess of the Sulfo-SMCC preparation was added to 83 picomoles of reporter DNA in water and incubated at room temperature for 30 minutes allowing the covalent conjugation of the amine-containing DNA with the *N*-hydroxysuccinimide (NHS) ester of Sulfo-SMCC by forming amide bonds. Excess crosslinker was removed using G-25 Sephadex column and the desalted Sulfo-SMCC linked reported DNA was immediately used for conjugation experiments with the detection antibodies.

Conjugation of Sulfo-SMCC linked DNA to antibodies was done by chemically reducing the disulfide bonds in IgG molecules using the modified approach of Joerger et al., (1995). For this purpose, we used an immobilized TCEP (Tris [2-carboxyethyl] phosphine hydrochloride) disulfide reducing gel (part # 77712) Thermo Fisher Scientific Inc., (Rockford, IL) which has several advantages such as it avoids rapid oxidation unlike other reducing agents such as dithiotreitol (DTT) and β-mercaptoethanol (BME) (Han and Han, 1994), easy recovery of the reduced antibodies, and no interference with common sulfhydryl-reactive reagents such as maleimide crosslinkers (Haugland and Bhalgat, 1998). Thus, the disulfide bonds of polyclonal anti-aflatoxin antibodies were reduced using the immobilized TCEP gel as per the manufacturer's instructions. Briefly, in a 1.5 ml microcentrifuge tube, 15 µl of the prewashed TCEP gel was mixed with 25 picomoles of detection antibody. This was done in 10 mM EDTA (Ethylenediaminetetraacetic acid) was further added to prevent oxidation of generated sulfhydryl groups. The reduction reaction was performed in ultrapure water for 15 minutes under gentle shaking and the tube was centrifuged at 1000 x g to obtain the supernatant fraction containing the reduced antibodies. The sulfhydryl-containing detector antibodies were immediately mixed with 95 µl of the desalted Sulfo-SMCC linked reporter DNA. While incubating in the dark for 30 minutes, the conjugation reaction was allowed to encourage formation of covalent linkage

between the malemide group of the SMCC molecules and the sulfhydryl groups of the TCEP reduced antibody molecules. Unbound molecules were further removed by buffer exchange using G-25 sephadex column. The antibody-DNA conjugate thus formed was used as the secondary detector antibody in further experiments.

Sandwich immunoassay and immuno magnetic bead recovery of aflatoxin.

A standard calibration curve was developed using dilutions of aflatoxin B1 captured with AFC-13 monoclonal anti-afaltoxin antibodies and PureProteome^{TM} protein G magnetic beads as solid supports in an optimized real time-PCR approach. Briefly, a standard working stock of 80 ppb aflatoxin B1 was prepared in ice cold 60:40 methanol:water solvent and two fold dilutions of aflatoxin B1 ranging from 0.3 to 10 ppb were prepared from this stock. Toxin capture was done using 1 μg (5 μl) of AFC-13 monoclonal anti-AFB1 antibodies in 1.5 ml microcentrifuge tubes containing 100 μl aflatoxin B1 standard for 5 minutes at room temperature with gentle shaking. A 10 μl suspension of PureProteome^{TM} protein G magnetic beads prepared in citrate-phosphate wash buffer (pH 5.0) was added and incubated at room temperature with gentle shaking carefully avoiding the settling of the beads. The citrate-phosphate wash buffer (pH 5.0) contained 4.7 g/L citric acid and 9.2 g/L dibasic sodium phosphate (Na_2HPO_4) dehydrate and it was used for washing and re-suspending the protein G beads. After a 15 min incubation, the solvent containing unbound molecules was aspirated using a pipette after allowing the magnetic beads to form a pellet using a magnet. The beads were further washed once with 200 μl of citrate-phosphate wash buffer and suspended in 25% normal rabbit serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) prepared in the same wash buffer used for blocking the beads. After a 5 min blocking step, 100 μl of detection antibody (reporter DNA conjugated polyclonal anti-AFB1 antibody at 0.0075 ng DNA/μl) diluted in wash buffer was added. A short incubation time of 5

min was used to reduce nonspecific binding of the detection antibody. Immediate recovery of the magnetic beads supporting a mouse anti-AFB1 monoclonal antibody-aflatoxin B1-rabbit anti-AFB1 polyclonal antibody sandwich was done by magnetic retention. Washing of the complex was done twice, each with PBS (0.01 M, pH 7.4) followed by 2-time nuclease-free water wash. After the final wash step, the beads were transferred to nuclease free 0.2 ml PCR tubes and heated for 10 min at 80^{o}C to release the bound molecules. After brief centrifugation at 5000 rpm, the supernatant was collected in new tubes and used for real time PCR detection.

Magnetic bead recovery and real time immuno-PCR analysis for the extractions of feed, feed grain and corn meal samples were done similarly. However, samples needed to be diluted further to avoid false negatives as explained below.

Detection and quantification of aflatoxin B1 using real time immuno quantitative-PCR.

The immuno-PCR assay was performed using an optimized assay using the MJ Research Opticon-2 real-time PCR detection system. Briefly, a 101-bp internal region of the reporter DNA was amplified in a 20 μl reaction mix containing 9.36 μl of template DNA and 1x concentration of PerfeCTa® SYBR® Green I FastMix® (Quanta BioSciences Inc., Gaithersburg, MD). Among three different primer pairs tested, an optimum pGL2B primer combination was used at 80 nM concentration of pGL2B forward (5'-GAACTGCCTGCGTCAGATTC-3') and pGL2B reverse (5'-AACCGTGATGGAATGGAACAAC-3'). Positive control of polyclonal anti-AFB1 antibody conjugated with DNA, antigen negative control that was processed similarly as the samples and a template negative control were included in each run. The optimized real-time PCR cycle parameters included a 95^{o}C initial denaturation step for 30 sec, followed by 40 cycles of denaturation (95^{o}C for 2 sec), and annealing (51^{o}C for 25 sec). Fluorescence measurements were taken after each annealing step. A melting curve analysis to detect potential formation of

nonspecific products was done from 50^{o}C to 90^{o}C with signal acquisition at every 0.2^{o}C melting rates for 1 sec hold time.

Aflatoxin detection and quantification in animal feeds and food samples was done using an optimized real time immuno quantitative-PCR sandwich immunoassay format (Fig. 1). A calibration curve was developed using several 2 fold dilutions of aflatoxin B_1 working stocks in 60:40 methanol:water solvent that were subjected to immunomagnetic bead based recovery and sandwich immunoassay (Fig. 2). During the amplification of reporter DNA, the increase in fluorescence signals after each PCR cycle was recorded to arrive at a threshold cycle value (C_t number, the cycle number where the fluorescence signal crosses a manually set threshold showing linear signal increase). The obtained C_t values were used to inversely correlate with antigen concentrations, and the template negative controls or antigen negative controls would have the highest numerical C_t values. Using the calibration curve, the aflatoxin content of samples was estimated by correlating C_t value of the recovered DNA to the standard toxin concentrations (Fig. 2).

Data analysis.

Statistical analysis of fluorescence signals and plotting of the amplification curves was done using SigmaPlot 11 software. Standard curve analysis was done using linear regression of four intra-assay replicates. Standard deviation values for cycle threshold numbers (Ct) were obtained by the opticon-2 software that computes the population standard deviation evaluated over the entire set of sample Ct values. Similarly, the aflatoxin contents (ppb) were calculated based on the linear regression equation and population standard deviations were calculated using MS Excel software.

Detection of aflatoxin using CD-ELISA.

For comparison purposes, the methanol extracts of mixed feeds, corn and yellow corn meal were subjected to aflatoxin test using a competitive direct enzyme linked immunosorbent assay (CD-ELISA) called 'Agri-Screen' (part # 8010) supplied by Neogen corporation (Lansing, MI). This test kit provides visual semi-quantitative estimation of total afaltoxins and is widely used by quality control personnel worldwide for several commodities including corn, peanuts, feed grains and mixed feeds. The Agri-Screen test kit has been approved from USDA's Grain Inspection, Packers and Stockyards Administration (USDA/GIPSA #2006-09) and Association of Analytical Communities (AOAC official method #990.32). The sample extracts were screened for aflatoxin against a known control concentration of 20 ppb aflatoxin following the manufacturer provided protocol. Briefly, a 100 µl of the thoroughly mixed sample extract (60:40 methanol:water) was combined with equal volume of enzyme- conjugated aflatoxin in a mixing well and added on to anti-aflatoxin antibodies immobilized in a microwell. The competitive reaction for the available antibody binding sites was allowed for two minutes between the free toxin in the sample (or control) and the added enzyme- conjugated toxin. The wells were washed for five times with deionized water and after the washing step, a 100 µl of substrate is added that reacts with the bound enzyme conjugate to produce blue color. After 3 minutes, 100 µl of stop solution was added to end the reaction and the resultant color of the sample and control were visually compared.

The aflatoxin wastes, toxin-spiked food/feed, and toxin-positive grain/feed samples was done by detoxifying or decontaminating with bleach according to the USDA guidelines prior to autoclaving and disposing as biohazard wastes.

Results and Discussion

Immuno-PCR optimization.

Development of the immuno-PCR (i-PCR) assay for aflatoxin detection in mixed feeds and feed grains involved initial optimization and troubleshooting mainly aimed at sensitive detection, signal amplification and minimizing nonspecific binding of DNA-conjugated secondary detection antibodies. The real-time PCR was optimized with gradient concentrations of forward and reverse primers amplifying an internal fragment of the reporter DNA in order to obtain higher signals in positive samples and to see no detectable fluorescence signal in negative control (nuclease free water). A primer concentration of 80nM was optimized for use with the PerfeCTa® SYBR® Green I FastMix® for better amplification efficiency and fast cycling. Each iPCR reaction included melting curve analyses to detect nonspecific products if any. The reaction efficiency was tested to meet quantification requirements of real-time PCR using dilutions of antibody conjugated reporter DNA.

Detection and quantification of aflatoxin in methanol extracts of mixed feeds, corn and yellow corn meal samples.

The complexity of food and feed matrices could affect the sensitivity of an immunoassay due to the interference of undefined and defined constituents of proteins, fats, and carbohydrates. These matrices may also affect the release of bound aflatoxin and hence the extraction methods invariably involve use of organic solvents and water along with mechanical shaking. A methanol:water mix is commonly used for feed ingredients and mixed feed extraction of aflatoxins. Use of water and hydroxylated solvents like methanol is highly recommended to

facilitate the effective release of aflatoxins into the extractant solution (Rayner and Dollear, 1968; Rayner et al., 1977). However, oftentimes analysis of feedstuffs involves several steps of extraction, filtration, sample cleanup, and concentration before subjecting to mycotoxin assays. The methodology described in this work requires a simple extraction step using 60% methanol in water and mechanical shaking without further time consuming sample cleanup. Use of antibody coated magnetic beads as solid support for sandwich immuno-PCR can be conveniently used for manual or semi-automatic bead recovery of mycotoxins using Dynal bead retriever. While developing a highly sensitive assay for this study, we used anti-aflatoxin B1 specific mouse monoclonal antibodies for toxin capture. The antibodies used in this study were unaffected by methanol solvent, and the toxin precipitation was rapidly done by immobilizing the toxin captured antibodies onto protein G magnetic beads. We found that the nonspecific binding of secondary antibodies onto the magnetic beads was much stronger and any harsh washing steps using nonionic detergents of Tween-20 or Triton X-100 oftentimes eliminated the specific binding of antigen-antibody complexes. This further compromised the assay sensitivity beyond 0.1 ppb of detection limits and 0.3 ppb of quantification limits. Thus, the use citrate phosphate (pH 5.0) buffer assisted both for antibody binding onto beads and washing to remove loosely bound constituents of feed or food matrices.

Examining the use of rapid and sensitive assay for aflatoxin quantification, we used our test strategies for determining aflatoxin in complex matrices of mixed feeds, feed ingredients and food commodities. For the first part of the study, the methanol extractions of the samples were first confirmed for aflatoxin content using the Agri-Screen test kit which is widely used and USDA/GIPSA approved for aflatoxin testing. However, due to the semi-quantitative nature of aflatoxin estimation from the competitive direct-ELISA using this kit, the actual concentrations of aflatoxin are not stated. Thus, all the samples were found to be contaminated with aflatoxin and visually estimated to be containing less than or around 20 ppb of aflatoxin.

The second part of the study involved estimation of aflatoxin contents in the samples with or without spiking by adding known concentrations of aflatoxin. The methanol: water extracts from mixed feeds, feed ingredient corn, and yellow corn meal samples were individually subjected to magnetic bead recovery of aflatoxin sandwiched between capture and DNA linked detection antibodies as shown in the illustrated immunoassay format (Fig. 1).

A high dose hook effect (Prozone) was observed while developing calibration curves using higher toxin levels beyond our detection limits. This is commonly seen in immunoassays involving antigen concentrations beyond upper detection limits (Rodbard et al., 1978; Fernando and Wilson, 1992; Killeen et al., 1993). Without regard for the hook effects, samples could potentially exhibit false negative results showing higher cycle threshold numbers (i.e., low toxin concentrations) in the real time PCR assay. In such cases, the use of sample dilution protocols is suggested (Saryan et al., 1989; Butch, 2000; Tang and Standage, 2000). Thus, at least two dilutions of four to ten-folds were adapted to detect the hook effect from samples containing unknown amounts of toxin levels.

Ideally, several dilutions should be done until a fluorescence signal curve comes later (higher cycle number) than that of the previous dilution assay during the PCR amplification indicating a weaker signal response concomitant with a lower sample level due to dilution. However, this strategy could add up the assay cost due to multiple measurements for one sample. Thus, a cost effective method of pooling the similar samples can be done to see if any of the pooled samples show false negative results due to hook effect (Cole et al., 1993).

We therefore opted to verify potential high aflatoxin concentrations and avoid possible false negatives by performing measurements using undiluted and diluted extracts as suggested by several researchers who have encountered the hook effect (Saryan et al., 1989; Butch, 2000; Tang and Standage, 2000). Thus, for samples containing unknown amounts of antigen in excess, initial

optimizations should be done to arrive at particular fold of dilutions required and can be standardized for routine estimations. Following this strategy, the fluorescence plots for undiluted and diluted extracts of samples were generated from the real time PCR amplification of the recovered reporter DNA. We first tried the immunoassay using 4 -fold diluted extracts and evidently, the PCR fluorescence plots of corn and yellow corn meal extracts (Figs. 3-5) showed poor resolution and failed to provide clear distinction from the signals of undiluted samples. This suggested that sample extracts need to be further diluted. The expected differences were then observed after correcting aflatoxin content (ppb) for dilutions in extracts from corn grain and yellow corn meal samples. Aflatoxin contents in corn and yellow corn meal sample extracts were quantified to be 3.12 ppb and 5.28 ppb respectively (Table 1 and Fig. 6). This can be attributed to the high efficiency (99.52%) of the real time PCR reaction that was optimized to distinguish even 2- fold difference in DNA templates. Thus, more than one dilution and respective dilution corrections can be done to precisely quantify toxin levels. On the other hand, a preliminary rough estimation of the aflatoxin content in the extracted samples can be done using simple assay kits such as the Agri-Screen kits used in this study. In the case of the extracts that we tested using the Agri-Screen semi-quantitative test kit, most of the samples showed aflatoxin levels around 20 ppb. Thus, in order to obtain distinguishable fluorescence signal plots, a 10-fold dilution for samples containing nearly 20 ppb aflatoxin and 40-fold dilution for samples containing up to 200 ppb aflatoxin was adapted.

Undiluted methanol extracts from mixed feeds, corn, and yellow corn meal samples were compared with respective 10- fold diluted extracts by subjecting them to bead recovery followed by real time immuno-PCR quantification to generate the fluorescence signals (Fig. 7). In the case of horse feed, dairy feed-1, dairy feed- 2, the signals from the diluted extracts showed higher cycle threshold numbers than the undiluted extracts (Figs. 8, 9, and 10 respectively) meaning that 10- fold dilutions were enough for accurate quantification in these samples. Further, the cycle

threshold numbers from the 10 -fold dilution signal plots were used to calculate aflatoxin content by the linear regression equation of the calibration curve. The aflatoxin content in horse feed, dairy feed-1 and dairy feed-2 were estimated to be 0.41 ppb, 4.25 ppb and 1.5 ppb respectively (Table 2 and Fig. 11). In the case of poultry feed, corn grain, and yellow corn meal extracts, excess antigen hook effects were clearly seen as the signals from the diluted extracts exhibited lower cycle threshold numbers than their undiluted counterparts. (Figs. 12, 13, and 14, respectively). If hook effects are ignored, this may lead to incorrect results estimating falsely lower aflatoxin levels than actual concentrations. Though, these samples were roughly estimated to be containing aflatoxin amounts of around 20 ppb using the Agri-Screen test kit, the high dose hook effect could be due to the presence of aflatoxin content above the detection limits of the immuno-PCR assay itself. As expected, the aflatoxin contents calculated using the cycle threshold numbers of the 10- fold diluted extracts of poultry feed, corn and yellow corn meal amounted to be 7.09 ppb, 16.34 ppb and 24.52 ppb respectively (Table 2 and Fig. 11). Clearly the aflatoxin contents in these samples were above or near 10 ppb which is the higher detection limit of our assay. As shown in Fig. 15, the accuracy of the aflatoxin determinations were further evidenced by the measured aflatoxin content of dairy feed-1 and horse feed extracts that showed no considerable change in final concentrations (i.e., no hook effect) unlike the estimations for corn, yellow corn meal, dairy feed-2 and poultry feed extracts.

Spike and recovery experiments were done to test the validity and accuracy of our immuno PCR assay by adding a known amount of aflatoxin B1.Finely ground samples of poultry feed, dairy feed-1, horse feed, and yellow corn meal were each spiked with 200 ppb aflatoxin B_1 standard and stabilized for 30 minutes before subjecting them to methanol extraction. The extracts from each spiked samples were diluted to 10- and 40- fold before subjecting them and their undiluted counterparts to bead recovery and real time immuno PCR quantification. The generated fluorescence signals were plotted as shown in Fig. 16. In the case of spiked poultry

feed, the fluorescence signal curve from the 40- fold diluted extracts showed higher cycle threshold values detecting the hook effect from the undiluted extracts (Fig. 17). For the spiked dairy feed-1 extracts, the fluorescence signal curve of undiluted extract was close to that of 10-folds diluted extract. The higher cycle threshold values of 40- fold diluted extracts indicated that 10- fold dilution was sufficient for this sample. For both the spiked poultry and dairy feed samples, the aflatoxin contents were around 18 and 9 ppb respectively (Table 3 and Fig. 19). However, for the spiked poultry and dairy feed-1 extracts, the recovery was only around 10% and 5 % respectively (Fig. 20) due to the excess antigen hook effect even at the 40- fold dilution of the extracts. Similar results were noticed in the case of yellow corn meal extracts wherein the fluorescence signal curve showed higher cycle threshold numbers than the undiluted extracts (Fig. 21) and the estimated aflatoxin content was found to be close to 14 ppb (Table 3 and Fig. 19). In the case of spiked horse feed extracts, the recovered aflatoxin was estimated to be close to the spiked amount of 200 ppb from the 40 -fold diluted extract indicating the highest aflatoxin recovery (Table 3 and Fig. 23). The undiluted and 10- fold diluted spiked horse feed extracts detected a high dose hook effect. Increasing trend of the recovery from the spiked horse feed and yellow corn meal extracts showed need of further dilutions (Fig. 23).

Due to the need of complying with regulatory agencies for aflatoxins along with their associated toxicities impacting human and animal health, there is a growing need of achieving safety and security of animal feeds and foodstuffs. Developing rapid, sensitive and specific analytical methods to accurately quantify aflatoxins present even at trace levels can effectively meet this demand. The real time immuno quantitative PCR (RT iq-PCR) method demonstrated in this study does not need sample cleanup and can readily detects commonly- encountered high dose effects and can be used for quantifying aflatoxin contents extracted using organic solvents provided several dilutions are used to identify hook effects should they be present. This method efficiently detected aflatoxin B1 as low as 0.1 ppb as lower detection limit (LOD) up to 10 ppb of

higher detection limits. We propose the use of dilution strategies for estimating excess aflatoxin contents. Thus the RT iq-PCR assay developed here is a simple and effective method for detecting and quantifying aflatoxins in complex matrices of foods and mixed feedstuffs and it offers several advantages of sensitivity, specificity and accuracy over traditional ELISA based methods.

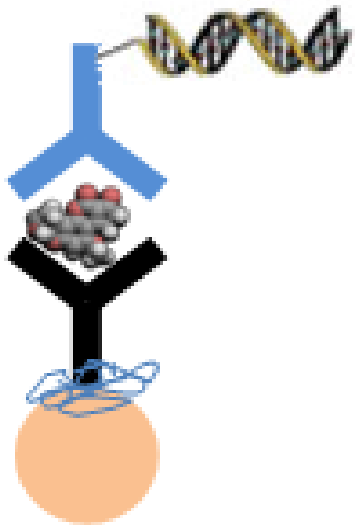


Figure 1. Illustration of the sandwich immunoassay format used in real time iq-PCR for detection and quantification of aflatoxins in animal feeds and feed grains. PureProteome® protein G beads (green) were used as solid supports, AFC-13 anti-AFB_1 mouse monoclonal antibodies (black) were used for toxin capture and A-8679 anti-aflatoxin polyclonal antibodies (blue) tethered with reporter DNA were used as detection antibodies.

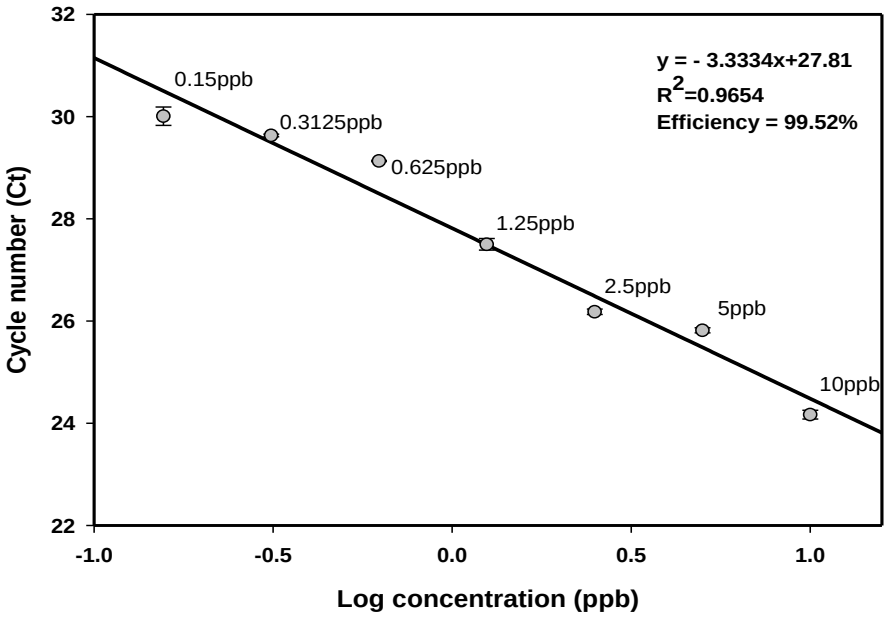


Figure 2. Calibration curve generated for detecting aflatoxins in animal feeds and feed grains. Intra-assay comparison of the three real time iq-PCR replicates of each standard was done to establish the reaction efficiency parameters.

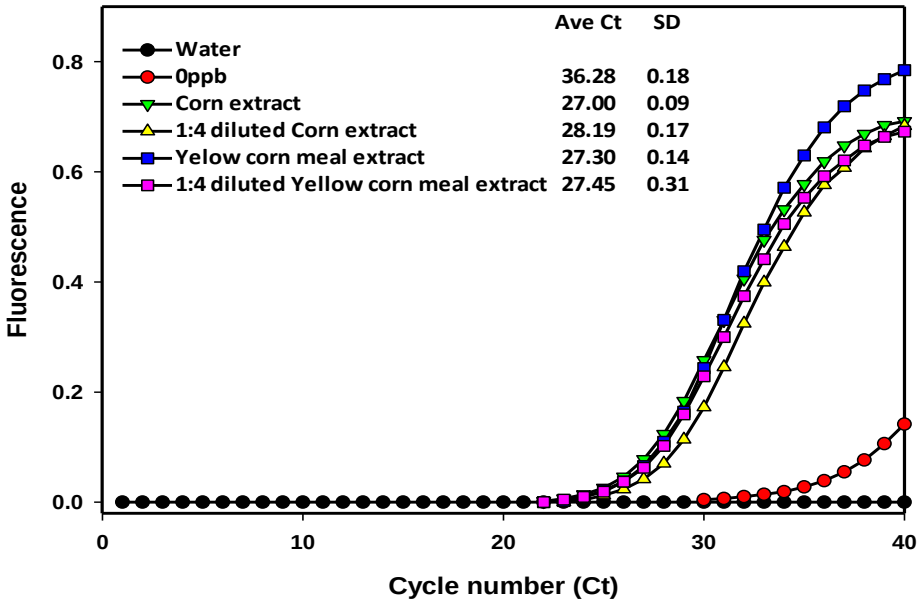


Figure 3. Immuno-PCR fluorescence signals from the 60% methanol extracts of corn grain and yellow corn meal extracts subjected to aflatoxin detection. Signals of undiluted extracts showed hook effect.

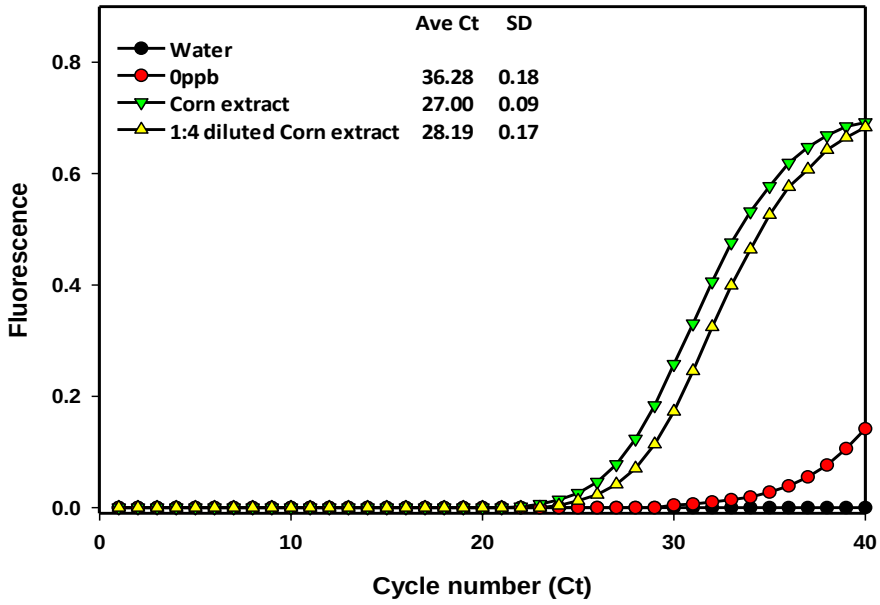


Figure 4. Immuno-PCR fluorescence signals from the 60% methanol extracts of corn feed grains subjected to aflatoxin detection immunoassay. Signals of undiluted extracts showed hook effect.

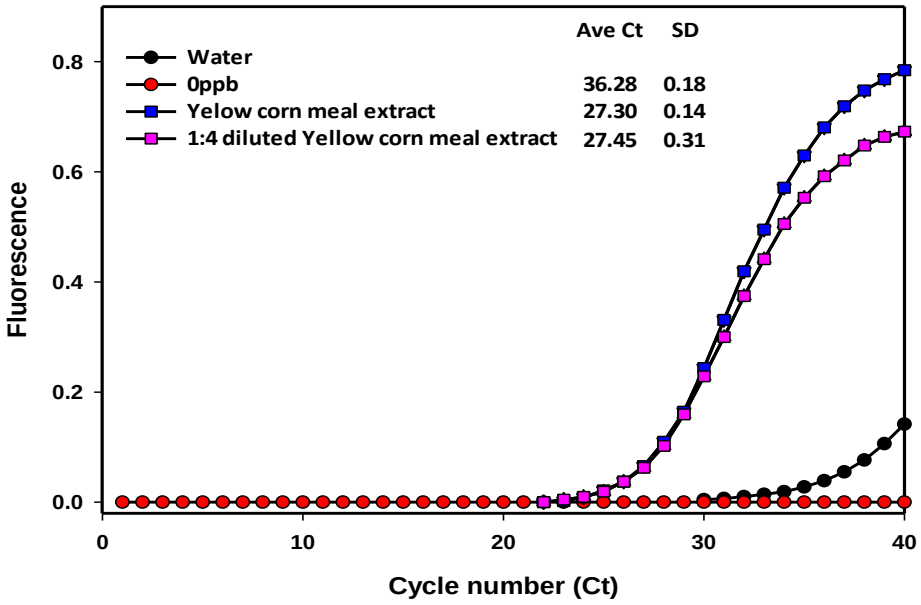


Figure 5. Immuno-PCR fluorescence signals from the 60% methanol extracts of yellow corn meal subjected to aflatoxin detection immunoassay. Signals of undiluted extracts showed hook effect.

Sample	Ave Ct	AFB1 (ppb)	SD (ppb)
CORN	27.00	1.77	0.12
1:4 CORN	28.19	3.12	0.36
YCM	27.31	1.43	0.14
1:4 YCM	27.45	5.28	1.11

Table 1. Aflatoxin content (ppb) in the undiluted and 1:4 diluted extracts of corn feed grain and yellow corn meal samples. Dilution corrected calculations of aflatoxin contents differed from estimations of the undiluted ones. Population standard deviations were calculated using MS excel from the aflatoxin content (ppb) of two sample replicates.

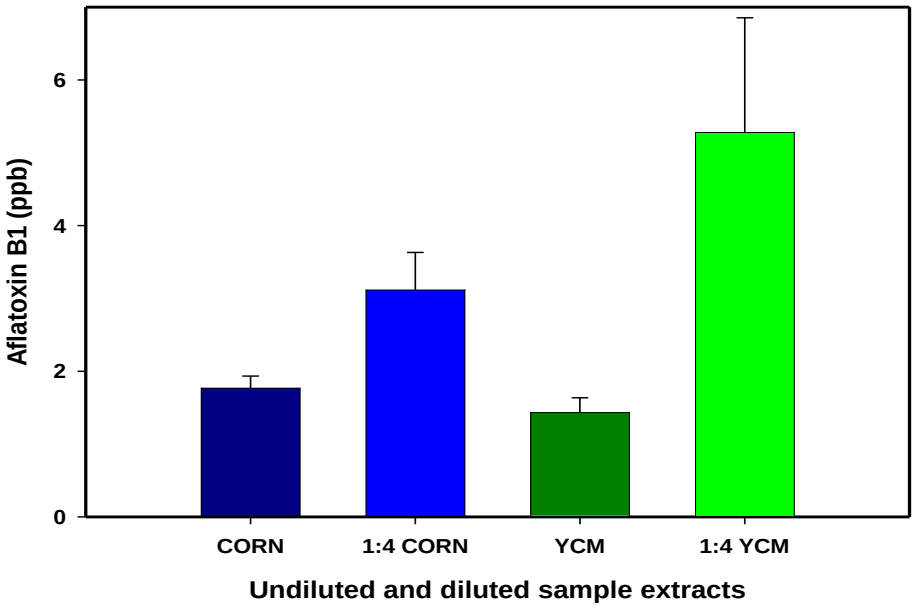


Figure 6. Bar charts of aflatoxin content (ppb) measured using undiluted and diluted extracts of corn feed grain and yellow corn meal samples. Error bars were calculated using two replicated sample means.

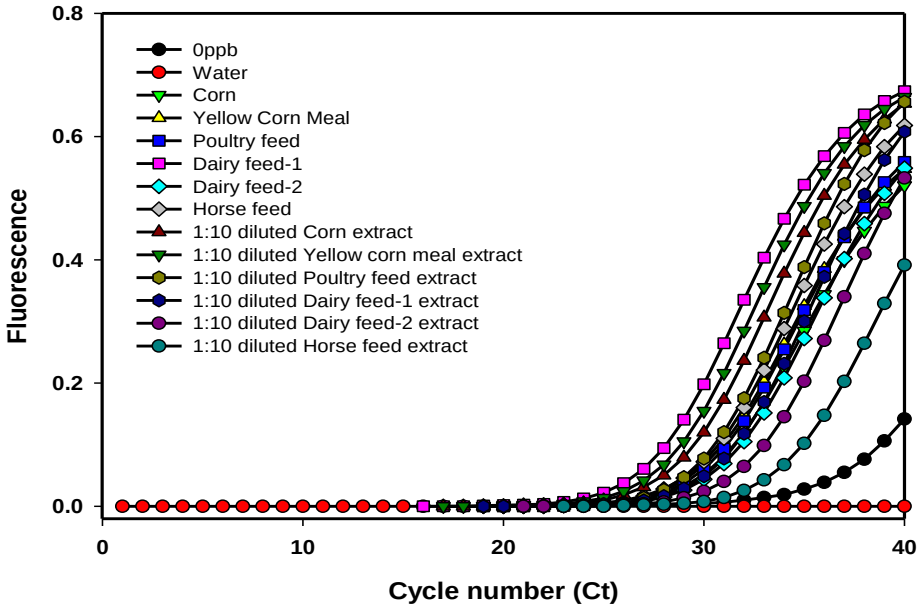


Figure 7. Immuno-PCR fluorescence signal curves from the animal feeds, corn and yellow corn meal extracts subjected to aflatoxin detection.

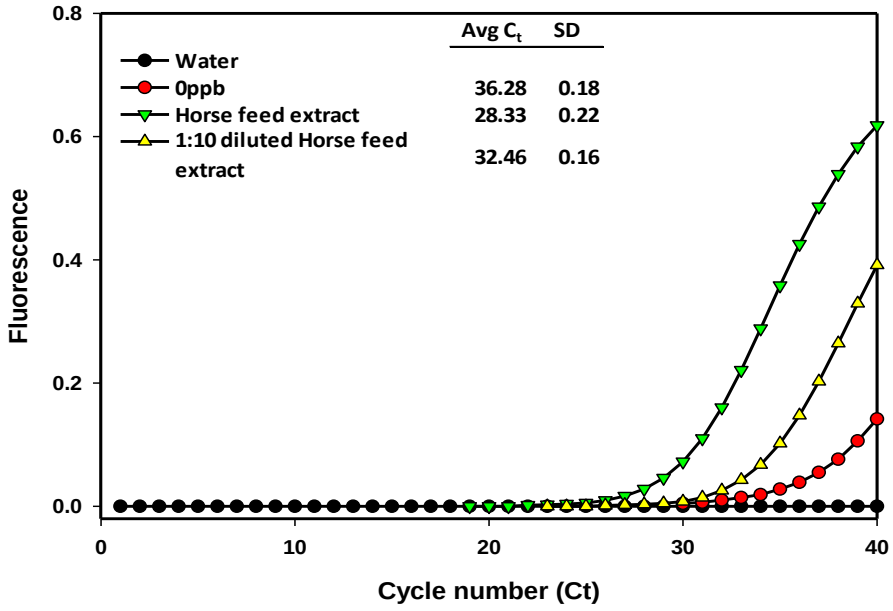


Figure 8. Immuno-PCR fluorescence signal curves from the horse feed extracts subjected to aflatoxin detection. Undiluted feed extracts did not show hook effect and signal curves differed from undiluted extracts.

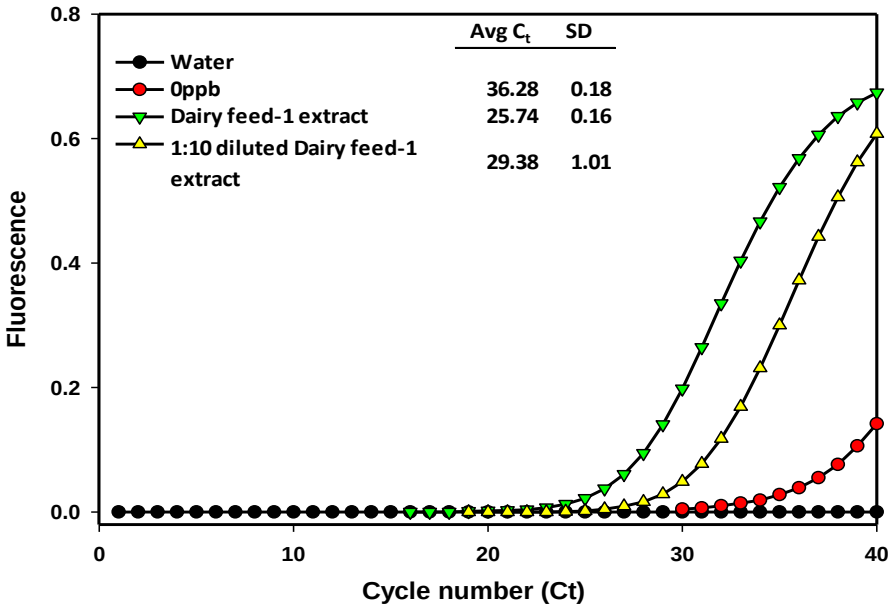


Figure 9. Immuno-PCR fluorescence signals from dairy feed-1 extracts subjected to aflatoxin detection. Fluorescence signal curves from the undiluted feed extracts were compared with 10-fold diluted extracts. The signal curves from 10-fold dilutions showed clear distinction from signal curves of the undiluted extracts.

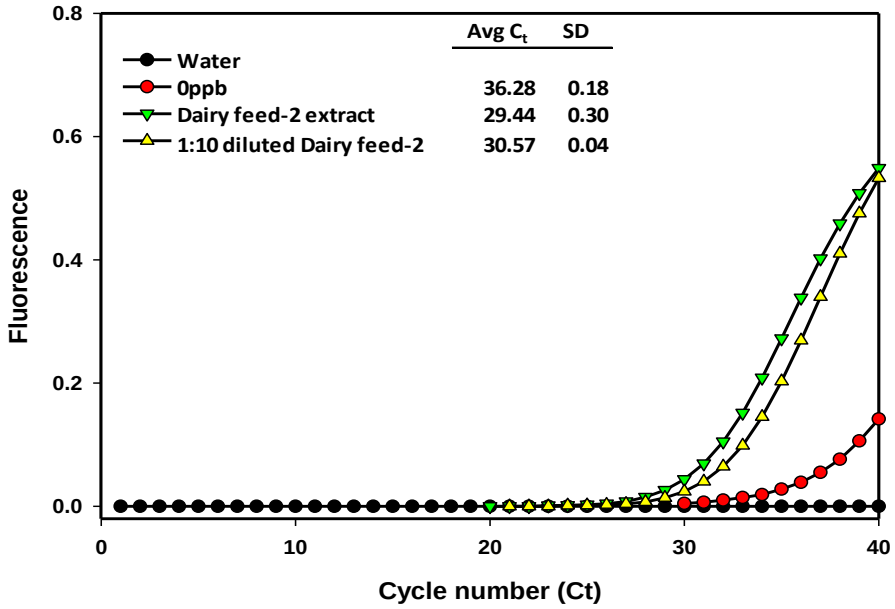


Figure 10. Immuno-PCR fluorescence signals from dairy feed-2 extracts subjected to aflatoxin detection. Fluorescence signal curves from the undiluted feed extracts were compared with 10-fold diluted extracts. The signal curves from 10-fold dilutions showed clear distinction from signal curves of the undiluted extracts.

Sample	AFB1 (ppb)	SD
CORN	0.43	0.06
1:10CORN	16.34	0.98
YCM	0.63	0.09
1:10YCM	24.52	1.55
Horse	0.71	0.11
1:10 Horse	0.41	0.04
Dairy-1	4.23	0.48
1:10 Dairy-1	4.25	2.57
Dairy-2	0.33	0.07
1:10 Dairy-2	1.50	0.05
Poultry	0.53	0.14
1:10 Poultry	7.09	0.51

Table 2. Aflatoxin content (ppb) in the undiluted and diluted extracts of mixed feeds, corn grain and yellow corn meal samples. Dilution corrected calculations of aflatoxin content from 10- fold dilutions of the sample extracts differed from estimations of the undiluted ones. Population standard deviations were calculated using MS excel from the aflatoxin content (ppb) of two sample replicates.

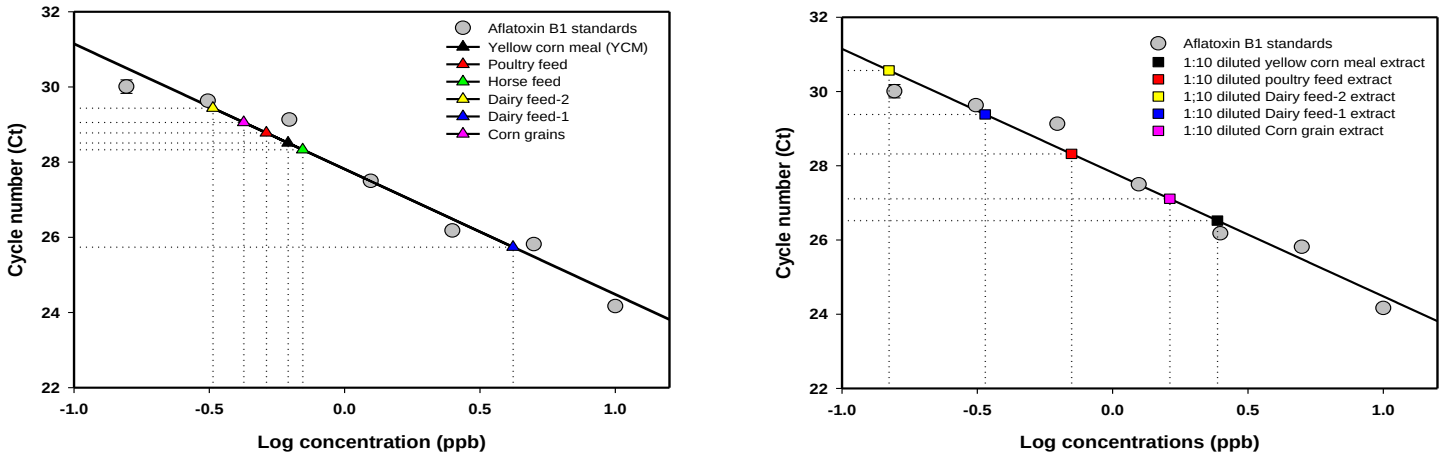


Figure 11. Quantification of aflatoxin B1 in animal feed, food grain and yellow corn meal extracts. Undiluted and diluted feed extracts were subjected to double antibody sandwich assay using PureProteome protein G beads. Undiluted feed extracts were compared with 10-fold diluted extracts.

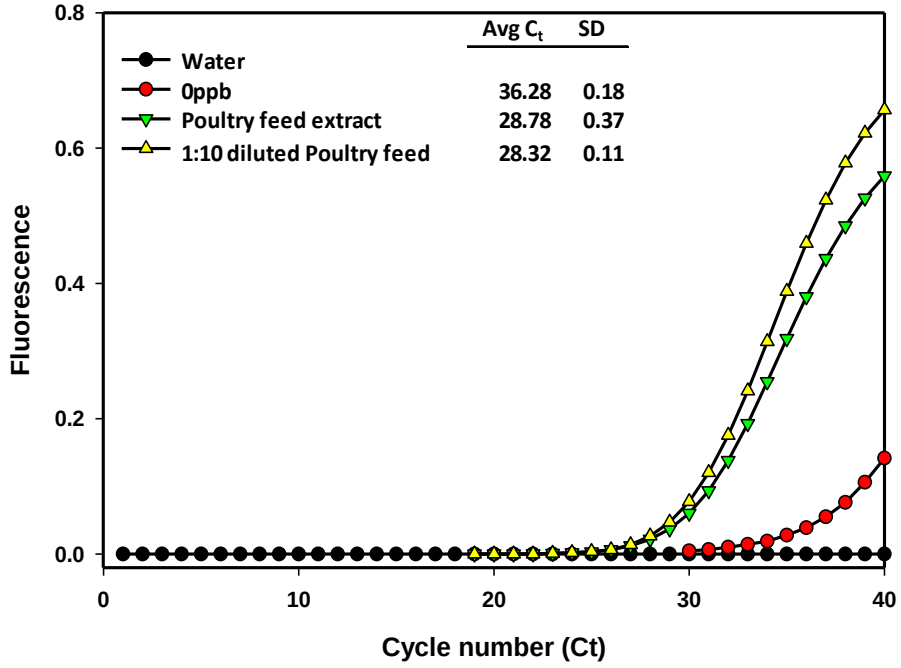


Figure 12. Comparison of aflatoxin detection in poultry feed extracts. Fluorescence signal curves from the undiluted feed extracts were compared with 10-fold diluted extracts to detect hook effect due to aflatoxin content beyond detection limits. The signal curves from 10-fold dilutions did not show clear distinction from signal curves of the undiluted extracts.

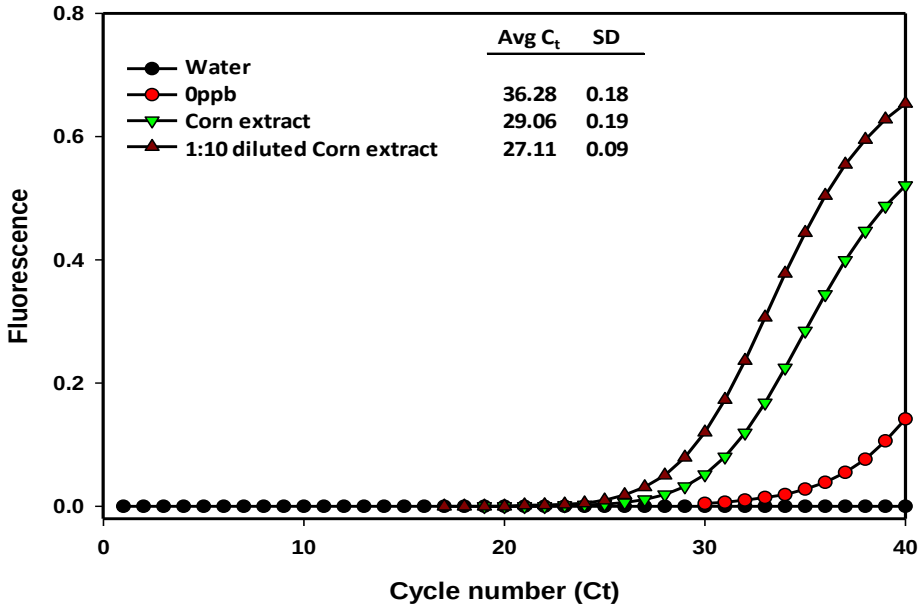


Figure 13. Comparison of aflatoxin detection in corn grain extracts. Fluorescence signal curves from the undiluted feed extracts were compared with 10-fold diluted extracts to detect hook effect due to aflatoxin content beyond detection limits. The signal curves from 10-fold dilutions showed clear distinction from signal curves of the undiluted extracts.

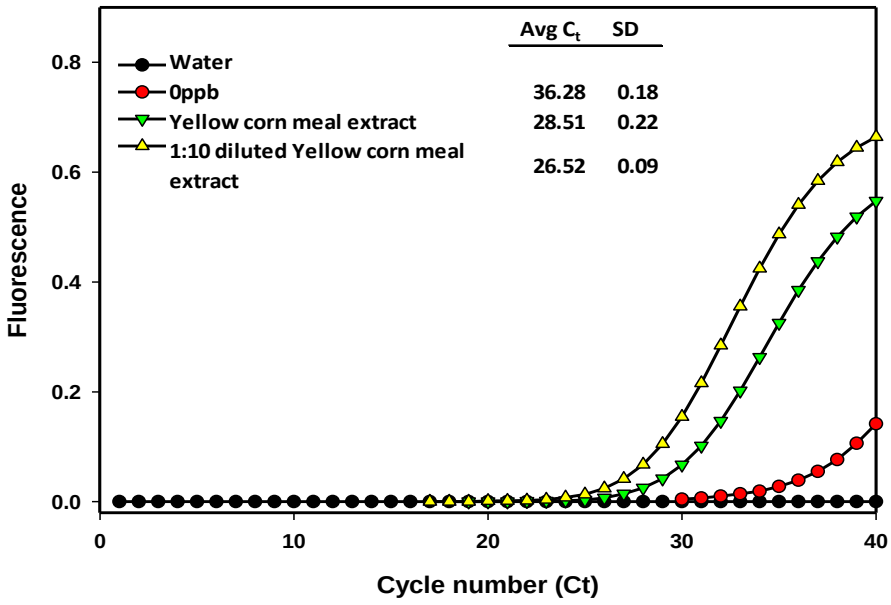
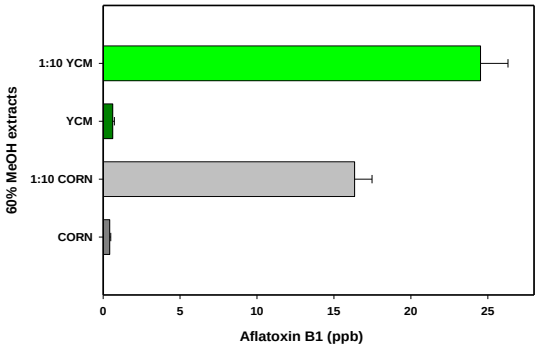
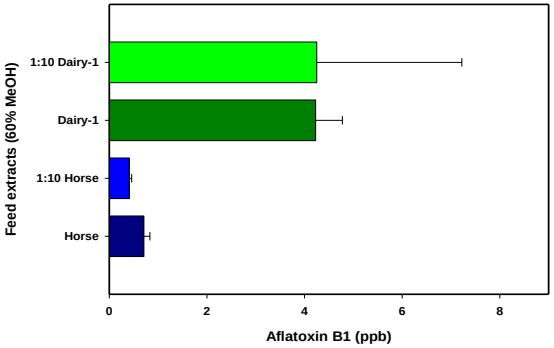


Figure 14. Comparison of aflatoxin detection in Yellow corn meal extracts. Fluorescence signal curves from the undiluted feed extracts were compared with 10-fold diluted extracts to detect hook effect due to aflatoxin content beyond detection limits. The signal curves from 10-fold dilutions showed clear distinction from signal curves of the undiluted extracts.





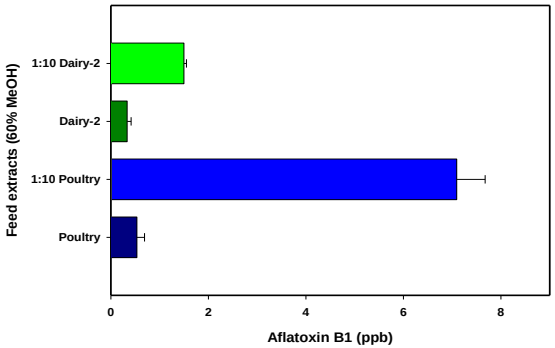


Figure 15. Bar chart illustrations from the aflatoxin quantified in mixed feeds, corn grains and yellow corn meal extracts. Diluted extracts of yellow corn meal, dairy feed-2 and poultry feed showed clear difference from the undiluted extracts. Dairy and horse feed extracts did not show considerable dilution effect.

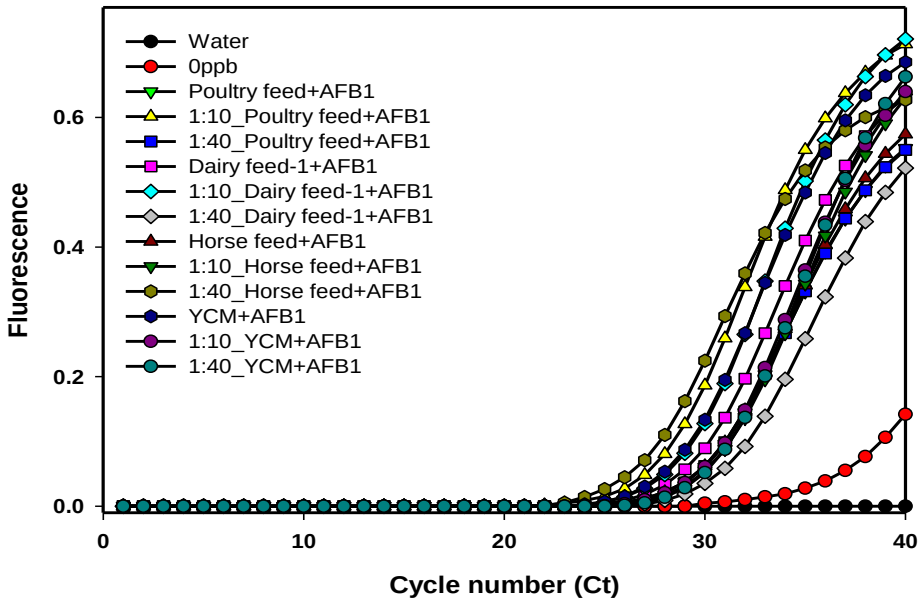


Figure 16. Fluorescene signal curves of aflatoxin detection from spiked (200 ppb AFB1) animal feeds, corn and yellow corn meal samples subjected to methanol extraction and double antibody sandwich assay using PureProteome protein G beads.

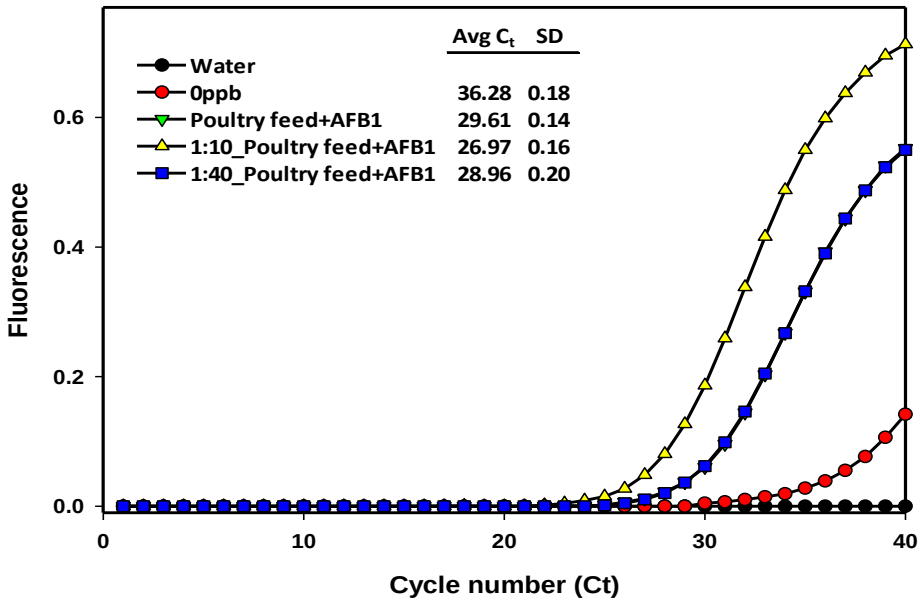


Figure 17. Spiked poultry feed extracts showing difference in fluorescence signal curves due to dilutions of extracts subjected for bead recovery and real time iq-PCR.

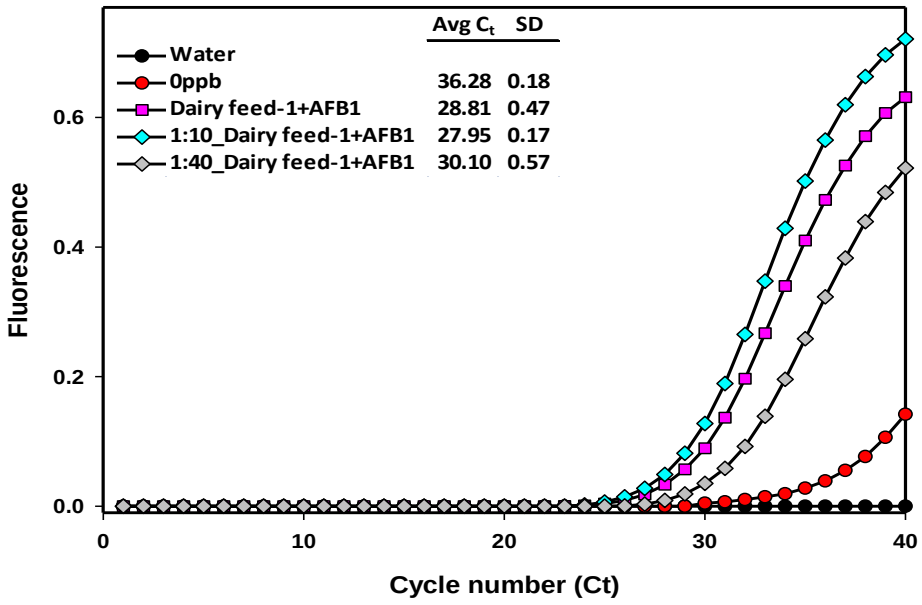


Figure 18. Spiked dairy feed-1 extracts showing difference in fluorescence signal curves due to dilutions of extracts subjected for bead recovery and real time iq-PCR.

AFB1 spiked Feed extracts (60% MeOH)	AFB1 (ppb)	SD
Poultry feed+AFB1	0.29	0.03
1:10_Poultry feed+AFB1	18.04	2.08
1:40_Poultry feed+AFB1	18.33	2.65
Dairy feed-1+AFB1	0.53	0.17
1:10_Dairy feed-1+AFB1	9.17	1.08
1:40_Dairy feed-1+AFB1	8.91	3.29
Horse feed-1+AFB1	0.29	0.07
1:10_Horse feed+AFB1	2.66	0.85
1:40_Horse feed+AFB1	194.38	14.92
YCM+AFB1	0.94	0.17
1:10_YCM+AFB1	3.19	0.68
1:40_YCM+AFB1	13.80	3.36

Table 3. Aflatoxin content (ppb) in the undiluted and 1:10, 1:40 diluted extracts of spiked mixed feeds, corn grain and yellow corn meal samples. Dilution corrected calculations of aflatoxin content from 10 and 40- fold dilutions of spiked poultry and dairy feed-1 extracts did not differ considerably unlike the spiked horse feed and yellow corn meal extracts. Population standard deviations were calculated using MS-excel from the aflatoxin content (ppb) of two sample replicates.

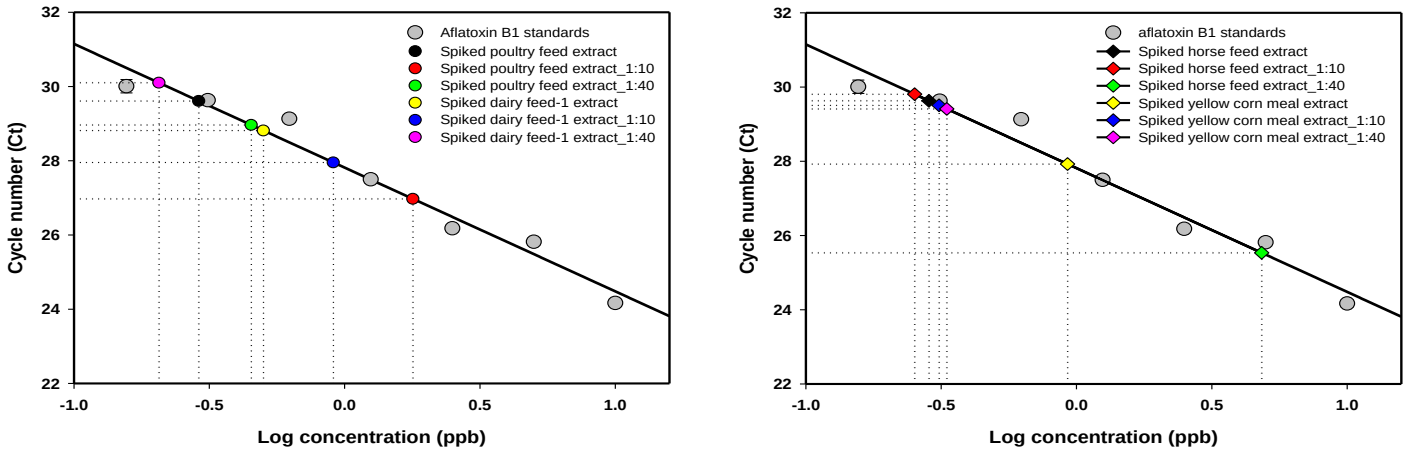


Figure 19. Aflatoxin B_1 recovery from the spiked feed and food grain samples. Undiluted feed extracts compared with 1:10 and 1:40 diluted extracts to detect excess antigen hook effect and to identify false negatives.

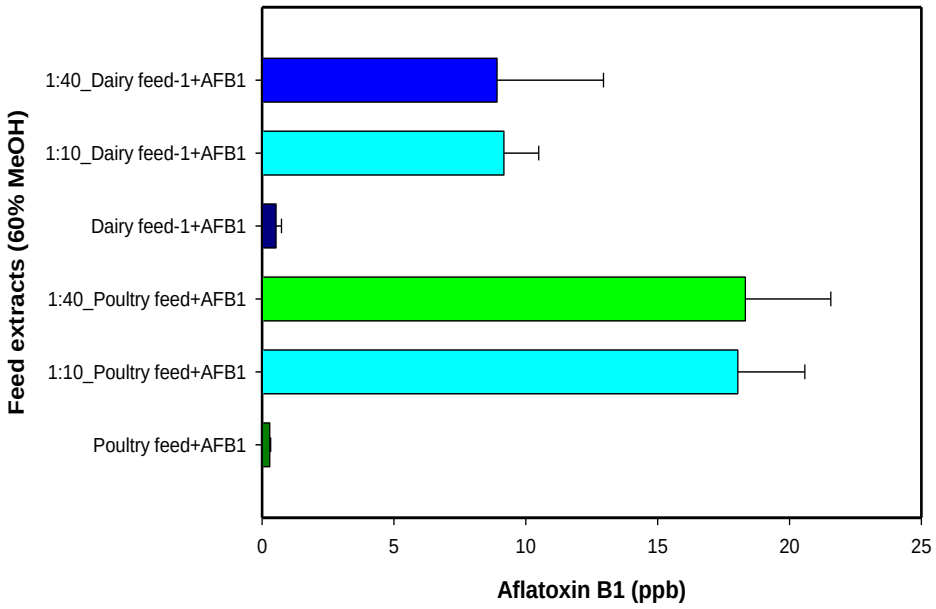


Figure 20. Bar chart illustrations from the aflatoxin quantified in spiked dairy and poultry feeds. The extracts did not show decreasing aflatoxin content due to dilutions.

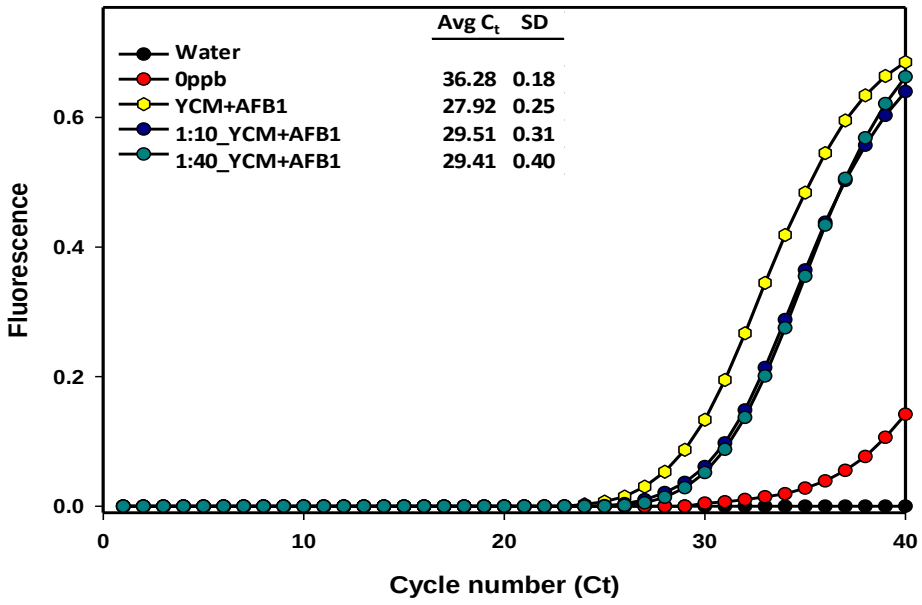


Figure 21. Fluorescence signal curves from the spiked yellow corn meal extracts. Undiluted and diluted extracts were subjected to immunoassay for aflatoxin detection. The diluted extracts showed hook effect due to aflatoxin content beyond detection limits.

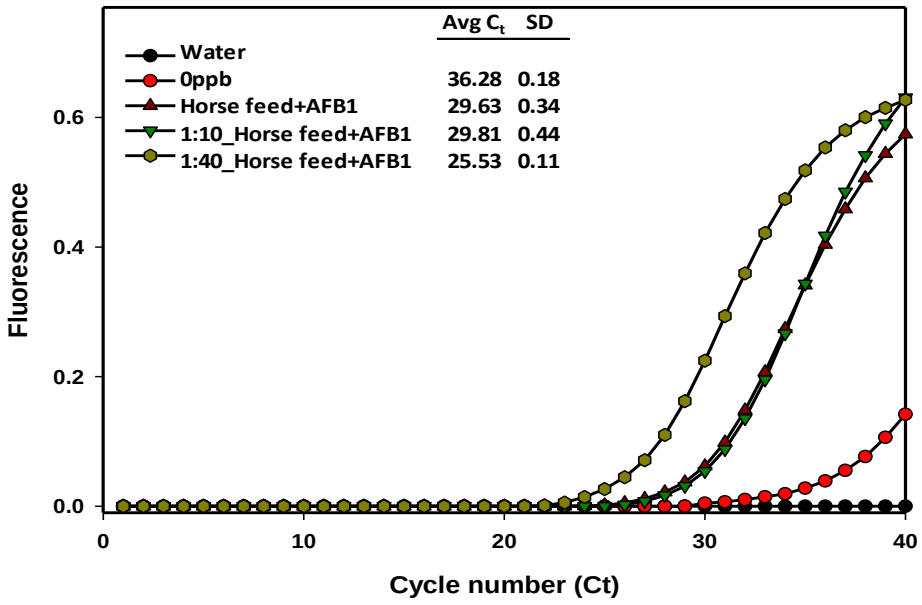


Figure 22. Fluorescence signals from the spiked horse feed extracts comparing undiluted and diluted extracts for aflatoxin detection. The 40-fold diluted extracts showed lower cycle threshold values due to hook effect from the 10 fold diluted and undiluted extracts containing possible aflatoxin concentrations beyond detection limits.

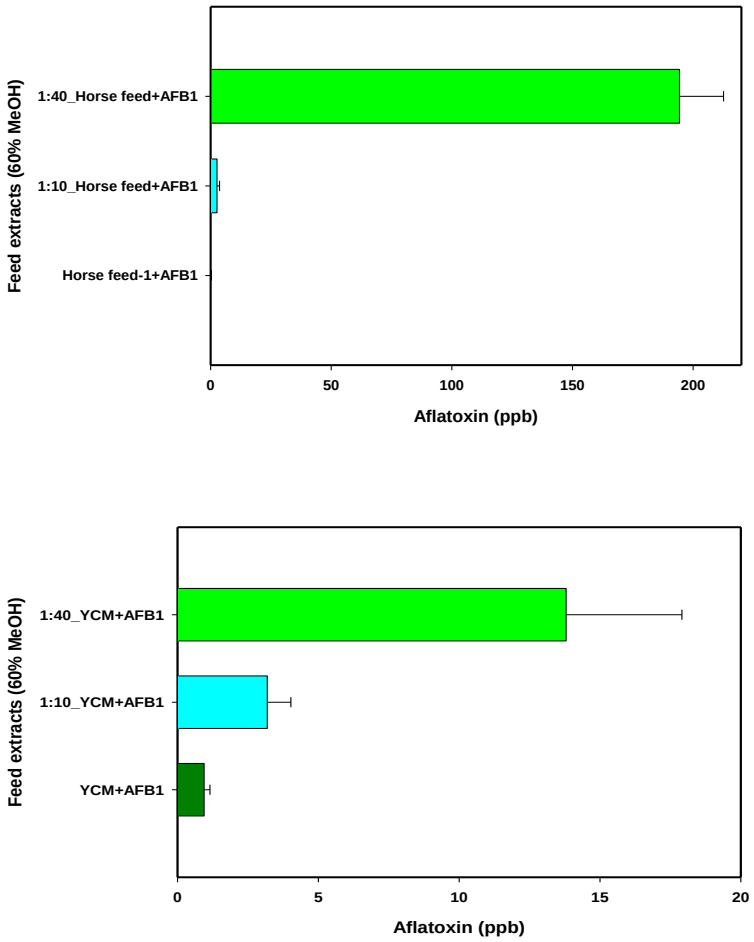


Figure 23. Bar chart illustrations from the aflatoxin quantified in spiked horse feed and yellow corn meal extracts. Increasing aflatoxin content in the diluted samples showed the importance of dilution to detect hook effect due to high aflatoxin concentration in undiluted extracts.

References

Abbas, H.K., Shier, W.T. and Cartwright, R.D. (2007) Effect of temperature, rainfall and planting date on aflatoxin and fumonisin contamination in commercial Bt and non-Bt corn hybrids in Arkansas. Phytoprotection 88, 41.

Adler, M., Wacker, R. and Niemeyer, C.M. (2003) A real-time immuno-PCR assay for routine ultrasensitive quantification of proteins. Biochemical and Biophysical Research Communications 308, 240.

Bennett, J.W. and Klich, M. (2003) Mycotoxins. Clin Microbiol Rev 16, 497.

Bhatnagar, D., Ehrlich, K.C. and Cleveland, T.E. (2003) Molecular genetic analysis and regulation of aflatoxin biosynthesis. Appl. Microbiol. Biotechnol. 61, 83.

Bircan, C., Barringer, S.A., Ulken, U. and Pehlivan, R. (2008) Increased aflatoxin contamination of dried figs in a drought year. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 25, 1400.

Butch, A.W. (2000) Dilution protocols for detection of hook effects/prozone phenomenon. Clinical Chemistry 46, 1719.

Casado, J.M., Theumer, M., Masih, D.T., Chulze, S. and Rubinstein, H.R. (2001) Experimental subchronic mycotoxicoses in mice: Individual and combined effects of dietary exposure to fumonisins and aflatoxin B_1. Food Chem. Toxicol. 39, 579.

CAST. (2003) Mycotoxins: Risks in plant, animal, and human systems. Task Force Report - Council for Agricultural Science and Technology, xvi + 199 pp.

Cole, T.G., Johnson, D., Eveland, B.J. and Nahm, M.H. (1993) Cost-effective method for detection of hook effect in tumor-marker immunometric assays. Clinical Chemistry 39, 695.

Cotty, P., Bayman, P., Egel, D. and Elias, K. (1994) Agriculture, aflatoxins and aspergillus. . In: R. Powell KA, A. and Peberdy, JF (Ed) The genus aspergillus: From taxonomy and genetics to industrial application. Plenum Press, New York, NY.

FAO (2004) Worldwide regulations for mycotoxins in food and feed in 2003. FAO Food Nutr. Pap., 1.

FAO/WHO (2008) Joint FAO/WHO expert committee report. *In: Animal feed impact on food safety,* FAO Headquarters, Rome, 8-12 October 2007., p. 54 pp.

Fernando, S.A. and Wilson, G.S. (1992) Studies of the hook effect in the one-step sandwich immunoassay. J. Immunol. Methods 151, 47.

Han, J.C. and Han, G.Y. (1994) A procedure for quantitative-determination of tris (2-carboxyethyl) phosphine, an odorless reducing agent more stable and effective than dithiothreitol. Anal. Biochem. 220, 5.

Harvey, R.B., Kubena, L.F., Elissalde, M.H., Corrier, D.E., Huff, W.E., Rottinghaus, G.E. and Clement, B.A. (1991) Cocontamination of swine diets by aflatoxin and diacetoxyscirpenol. J Vet Diagn Invest 3, 155.

Haugland, R.P. and Bhalgat, M.K. (1998) Preparation of avidin conjugates. Methods in Molecular Biology; Immunochemical protocols, Second edition, 185.

Hussein, H.S. and Brasel, J.M. (2001) Toxicity, metabolism, and impact of mycotoxins on humans and animals. Toxicology 167, 101.

IARC (1993) Toxins derived from fusarium moniliforme: Fumonisins B_1 and B_2 and fusarin C. IARC Monogr Eval Carcinog Risks Hum 56, 445.

IARC (1993) Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. IARC monographs on the evaluation of carcinogenic risks to humans. In, Vol. 56. International Agency for Research on Cancer (IARC) Working Group, World Health Organization, Lyon, France, p. 467.

Joerger, R.D., Truby, T.M., Hendrickson, E.R., Young, R.M. and Ebersole, R.C. (1995) Analyte detection with DNA-labeled antibodies and polymerase chain-reaction. Clinical Chemistry 41, 1371.

Jones, F.T., Hagler, W.H. and Hamilton, P.B. (1982) Association of low-levels of aflatoxin in feed with productivity losses in commercial broiler operations. Poult Sci 61, 861.

Killeen, A.A., Ramey, M.L. and Dean, J.J. (1993) High-dose hook effect in an immunoluminometric thyrotropin assay - the open-faced sandwich artifact. Annals of Clinical Biochemistry 30, 413.

Leeson, S., Diaz , G.J. and Sumers, J.D. (1995) Poultry metabolic disorders and mycotoxins. University books, Guelph, ON, Canada.

Mannon, J. and Johnson, E. (1985) Fungi down on the farm. New Scientist, 12.

Meissonnier, G.M., Laffitte, J., Loiseau, N., Benoit, E., Raymond, I., Pinton, P., Cossalter, A.M., Bertin, G., Oswald, I.P. and Galtier, P. (2007) Selective impairment of drug-metabolizing enzymes in pig liver during subchronic dietary exposure to aflatoxin B_1. Food Chem. Toxicol. 45, 2145.

Miazzo, R., Peralta, M.F., Magnoli, C., Salvano, M., Ferrero, S., Chiacchiera, S.M., Carvalho, E.C., Rosa, C.A. and Dalcero, A. (2005) Efficacy of sodium bentonite as a detoxifier of broiler feed contaminated with aflatoxin and fumonisin. Poult Sci 84, 1.

Niemeyer, C. and Blohm, D. (1996) Protein detection with PCR (immuno-PCR). Nachrichten Aus Chemie Technik Und Laboratorium 44, 481.

Niemeyer, C.M., Adler, M. and Wacker, R. (2005) Immuno-PCR: High sensitivity detection of proteins by nucleic acid amplification. Trends in Biotechnology 23, 208.

Niemeyer, C.M., Adler, M. and Wacker, R. (2007) Detecting antigens by quantitative immuno-PCR. Nature Protocols 2, 1918.

Oliveira, C.A., Rosmaninho, J.F., Castro, A.L., Butkeraitis, P., Reis, T.A. and Correa, B. (2003) Aflatoxin residues in eggs of laying Japanese Quail after long-term administration of rations containing low levels of aflatoxin B_1. Food Addit Contam 20, 648.

Puschner, B. (2002) Mycotoxins. Vet Clin North Am Small Anim Pract 32, 409.

Rauber, R.H., Dilkin, P., Giacomini, L.Z., Araujo de Almeida, C.A. and Mallmann, C.A. (2007) Performance of turkey poults fed different doses of aflatoxins in the diet. Poult. Sci. 86, 1620.

Rayner, E.T. and Dollear, F.G. (1968) Removal of aflatoxins from oilseed meals by extraction with aqueous isopropanol. J Am Oil Chem Soc 45, 622.

Rayner, E.T., Koltun, S.P. and Dollear, F.G. (1977) Solvent-extraction of aflatoxins from contaminated agricultural products. Journal of the American Oil Chemists Society 54, A242.

Reddy, B.N., Brijitha, N. and Raghavender, C.R. (2005) Aflatoxin contamination in insect damaged seeds of horsegram under storage. Mycotoxin Res. 21, 187.

Richard, J.L. (2007) Some major mycotoxins and their mycotoxicoses-an overview. Int. J. Food Microbiol. 119, 3.

Riley, R.T., Kemppainen, B.W. and Norred, W.P. (1985) Penetration of aflatoxins through isolated human epidermis. J Toxicol Environ Health 15, 769.

Rodbard, D., Feldman, Y., Jaffe, M.L. and Miles, L.E.M. (1978) Kinetics of 2-site immunoradiometric (sandwich) assays. 2. Studies on nature of high-dose hook effect. Immunochemistry 15, 77.

Sano, T., Smith, C.L. and Cantor, C.R. (1992) Immuno-PCR - very sensitive antigen-detection by means of specific antibody-DNA conjugates. Science 258, 120.

Saryan, J.A., Garrett, P.E. and Kurtz, S.R. (1989) Failure to detect extremely high-levels of serum IgE with an immunoradiometric assay. Annals of Allergy 63, 322.

Tang, M.E. and Standage, J.C. (2000) Dilution protocols for detection of hook effects/prozone phenomenon. Clinical Chemistry 46, 1720.

Theumer, M.G., Canepa, M.C., Lopez, A.G., Mary, V.S., Dambolena, J.S. and Rubinstein, H.R. (2009) Subchronic mycotoxicoses in wistar rats: Assessment of the in vivo and in vitro genotoxicity induced by fumonisins and aflatoxin B_1, and oxidative stress biomarkers status. Toxicology 268, 104.

Trenk, H.L. and Hartman, P.A. (1970) Effects of moisture content and temperature on aflatoxin production in corn. Appl Microbiol 19, 781.

Vainio, H., Heseltine, E. and Wilbourn, J. (1994) Priorities for future IARC monographs on the evaluation of carcinogenic risks to humans. Environ Health Perspect 102, 590.

Vardon, P., McLaughlin, C. and Nardinelli, C. (2003) Potential economic costs of mycotoxins in the united states. In: Council for Agricultural Science and Technology (CAST). *Mycotoxins: Risks in Plant, Animal, and Human Systems.* Task Force Report No. 139, Ames, Iowa.

Wu, H.C., Huang, Y.L., Lai, S.C., Huang, Y.Y. and Shalo, M.F. (2001) Detection of *Clostridium botulinum* neurotoxin type a using immuno-PCR. Lett. Appl. Microbiol. 32, 321.

VITA

DINESH BABU

Candidate for the Degree of Doctor of Philosophy

Dissertation: RAPID AND SENSITIVE DETECTION OF AFLATOXIN IN ANIMAL FEEDS AND FOOD GRAINS USING IMMUNOMAGNETIC BEAD BASED RECOVERY AND REAL-TIME IMMUNO QUANTITATIVE-PCR (RT-iqPCR) ASSAY.

Major Field: Food Science

Biographical:

Education:

Bachelor of Science in Agriculture
University of Agricultural Sciences, Dharwad, India (1994).

Master of Science in Agricultural Microbiology
University of Agricultural Sciences, Bangalore, India (1997).

Master of Science in Soil Microbiology and Biochemistry
Oklahoma State University, Stillwater, OK, USA (2005).

Completed the requirements for the Doctor of Philosophy in Food Science/Food Microbiology at Oklahoma State University, Stillwater, Oklahoma in January, 2010.

Experience:

Graduate Research Assistant (Aug. 2008-2010)
Robert M Kerr Food and Agriculture Products Center, OSU, Stillwater, OK
Research Specialist (Oct. 2005-Aug. 2008)
Dept. of Microbiology and Molecular Genetics, OSU, Stillwater, OK
Graduate Research Assistant (June 2002-May 2005)
Oklahoma State University, Stillwater, OK
Project Assistant (March 2001-May 2002)
Indian Institute of Science, Bangalore, India
Research Associate-Microbiologist (July 1998-Jan 2001)
E I D Parry (I) Ltd., R&D Center, Bangalore, India
Project Assistant (August 1997-July 1998)
Indian Institute of Science, Bangalore, India

Professional Memberships:
Institute of Food Technologists (IFT)

Name: Dinesh Babu Date of Degree: December, 2010

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: RAPID AND SENSITIVE DETECTION OF AFLATOXIN IN ANIMAL FEEDS AND FOOD GRAINS USING IMMUNOMAGNETIC BEAD BASED RECOVERY AND REAL-TIME IMMUNO QUANTITATIVE-PCR (RT-iqPCR) ASSAY.

Pages in Study: 139 Candidate for the Degree of Doctor of Philosophy

Major Field: Food Science

Scope and Method of Study:
Aflatoxins are one of the major mycotoxins that are considered as unavoidable natural toxins encountered in food and feedstuffs. Aflatoxins are produced by several strains of *Aspergillus* fungi and known to be deleterious to human and animal health even at trace levels. Development of highly sensitive analytical methods for detection of aflatoxins could prevent potential health hazards while being in compliance with regulatory limits for the trade of commodities. In this study, we have developed a hitherto unexplored use of real time immuno quantitative PCR (RT iq-PCR) assay for sensitive detection and quantification of aflatoxins in animal feeds, feed grains and foodstuffs. The use of several immuno-PCR approaches to estimate aflatoxin contents in various agricultural commodities is outlined in this work. Our methodology can detect and quantify aflatoxins as low as 0.1 ppb in methanol extractions of complex food and feed matrices.

Findings and Conclusions:
The first part of the immuno-PCR approach developed here involves magnetic bead based recovery of aflatoxin B_1 (AFB_1) from methanol extractions of food and feed samples and sensitive quantification of AFB_1 using real time PCR. The use of different types of magnetic beads as solid supports for the immunoassays was tested to arrive at a bead type showing least nonspecific binding of signal generating marker. Development strategies for reporter DNA labels, conjugation of reporter DNA to secondary detection antibodies and performance of capture antibodies during immunoassays are outlined. We also tested usefulness of different monoclonal and polyclonal antibodies in direct and indirect sandwich assay formats as capture antibodies and verified the preference of detection antibodies tethered with reporter DNA marker. Calibration curves using several dilutions of aflatoxin standards in 60% methanol were developed. Second part of the study involved demonstration of the optimized immuno-PCR approach for detection and quantification of aflatoxins extracted from complex matrices of food and feedstuffs. Methanol extractions of animal feeds, corn feed grains and yellow corn meal were compared and most of the samples tested contained total aflatoxin content of less than 20 ppb. However, an excess antigen hook effect due to high analyte concentrations beyond the detection limit of our immuno-PCR assay (0.1 to 10ppb) was noticed as common occurrence in test samples. To overcome false negative results due to excess aflatoxin contents, we demonstrated the use of dilution protocols enabling the detection of very high levels of aflatoxins in feed extractions. Testing for the reliability of the immuno-PCR assay, samples were spiked with 200 ppb of aflatoxin B_1 and about 96% of the spiked aflatoxin was recovered from horse feed extracts which further demonstrated the assay performance and reliability. Considering the significance of estimating trace levels of aflatoxins and their serious implications on animal and human health, we thus developed and tested our quantitative real time immuno-PCR method that could be used as a model system for aflatoxin detection in complex matrices of food or animal feed samples.

ADVISER'S APPROVAL: _____