High Prevalence of Aflatoxin B\textsubscript{1} in *Aspergillus flavus* Infecting Stored Rice Grains

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**ABSTRACT**

One of the best-known rice-infecting microorganisms is *Aspergillus flavus*, which produces toxic metabolites known as Aflatoxins (AFs). This study was designed to detect potential simultaneous biosynthesis of the four main AFs (AFB\textsubscript{1}, AFB\textsubscript{2}, AFG\textsubscript{1}, and AFG\textsubscript{2}) in rice-infecting strains of *A. flavus*. The AF prevalence was studied in 109 strains of *A. flavus*, which were collected from stored Indian rice grains from 300 locations in the Middle Euphrates region of Iraq from 2015 to 2016. The potential AFs were extracted and quantified simultaneously using High-Performance Liquid Chromatography (HPLC) equipped with a photodiode array detector. The results revealed that 29% (n= 32) of strains were non-aflatoxigenic, while the remaining 71% (n= 77) were confirmed to be aflatoxigenic, with variable ability to produce mono-, bi-, and tri-AFs. AFB\textsubscript{1}, AFG\textsubscript{2}, AFG\textsubscript{1}, and AFB\textsubscript{2} were produced by 49 (45%), 44 (40%), 20 (18%), and 17 (16%) strains, respectively, at various concentrations. The concentration of AFB\textsubscript{1} was the highest among the *A. flavus* strains, with a mean value of 3,561.9 µg kg\textsuperscript{-1}. In conclusion, the most abundant AF synthesized by the rice-infecting *A. flavus* strains was AFB\textsubscript{1}. Contamination with AFs continues to pose potential health risks to animals as well as humans. These results clearly indicate that the improper storage conditions of rice in Iraq were favourable for the growth of *A. flavus* and contamination with AFs. National-level studies are mandatory to avoid foodborne intoxications. Strict regulations should be devised and imposed to prevent synthesis of AFs on rice grains.

**Keywords:** Aflatoxicogenic, Contamination, Indian rice, HPLC, Hygiene.

**INTRODUCTION**

Aflatoxins (AFs) are toxic secondary metabolites that are mainly synthesised by *Aspergillus flavus*, *A. parasiticus*, and *A. nomius*, which infect several economically important crops (Al-Shuhaib et al., 2018; Hasanvand et al., 2016; Heshmati et al., 2019). The growth of aflatoxicogenic *A. flavus* strains on certain foods and feeds may result in production of AFs, which can cause illness or lead to the death of humans and animals; therefore, it is an important public health concern (Aydin et al., 2007; Giray et al., 2009; Heshmati et al., 2019). The consumption of aflatoxigenic *A. flavus* strains infecting grains and other agricultural products is a serious public health issue worldwide. Therefore, several preventive and mitigation strategies have been proposed to control AF contamination in grains and other agricultural products. The exhaust of aflatoxigenic strains of *A. flavus* is feasible only when the appropriate conditions are met (Aydin et al., 2007; Giray et al., 2009; Heshmati et al., 2019). The prevalence and concentration of AFs in grains are supposed to be related to the storage environment and conditions (Aydin et al., 2007; Giray et al., 2009; Heshmati et al., 2019).

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At present, about 20 AFs have been identified, but those most frequently found in foods like cereals, rice, and corn are AFB₁, AFB₂, AFG₁, and AFG₂ (Li et al., 2014), of which AFB₁ is the most toxic (Erkmen and Bozoglu, 2008). Due to their toxic and carcinogenic potential, AFs have garnered considerable attention. Moreover, AFs are the most hazardous and mutagenic natural substances (Paterson and Lima, 2009). AFs are associated with aflatoxicosis in a variety of mammals, birds, and fishes (Frisvad et al., 2006); hepatocytes are believed to be the primary target cells (Towner et al., 2000). AFs are generally classified as group I carcinogenic substances by the International Agency of Research on Cancer (IARC), with a particular emphasis on AFB₁ (IARC, 1993). The increasing prevalence of AF contamination in rice has become a major concern for the scientific community because it poses a public health threat. Several rice-based dishes are essential components of daily meals in the Middle East and Indian subcontinent, which are well known for having high rice consumption. During storage, mycotoxigenic mould may grow on rice and cause mycotoxicosis when ingested (Villers, 2014). Rice represents an attractive substrate for fungal growth and toxinogenesis, and can be used as an ideal growth medium to investigate the potential toxicity of fungal strains (Bars and Bars, 1992). Previous studies have reported high natural levels of AFs in stored rice samples (Tanaka et al., 2007). Recently, high levels of AF contamination by moulds due to improper storage have been widely reported (Al-Zoreky and Saleh, 2017; Tournas and Niazi, 2018; Majeed et al., 2018). Development of AF contamination during storage is largely due to delayed crop mortality and excessive moisture, which is relatively high in tropical countries with high temperature and humidity (Cho et al., 2008; Rahman et al., 2017). In addition to climatic conditions, storage conditions of rice can exert a considerable influence on the fungal load and AF contents of the rice. Therefore, examination of stored rice grains intended for human consumption is necessary to determine the risk of AFs for consumers. Several countries have introduced strict guidelines regarding AF contamination in food commodities. Given the widespread occurrence of AF-producing fungi and AFs in some crops, several robust biological and chemical methods have been developed for AF detection. However, these methods can be time-consuming (Zheng et al., 2016) and require specialized expertise (Sirhan et al., 2014) or expensive enzymatic preparations (Xiao-han et al., 2017). High-Performance Liquid Chromatography (HPLC) coupled to a fluorescence detector remains the most versatile technique due to its high sensitivity and relatively low cost (Wen et al., 2013; Mashak et al., 2016). The present study was conducted to identify and quantify the AFs from A. flavus isolated from stored rice grains, and to estimate the pattern and severity of mycotoxin contamination in the rice. To the best of our knowledge, data concerning AF production in rice-contaminating A. flavus strains from Iraq are limited; therefore, the present study constitutes the first large-scale qualitative and quantitative investigation of naturally biosynthesized AFs in this country.

MATERIALS AND METHODS

Rice-Grain Sources and Isolation of A. flavus

A total of 300 Indian rice samples were purchased at random from local markets in several Middle Euphrates regions in Iraq during 2015–2016. Among the microorganisms observed in the cultured rice grains, 109 fungal strains of A. flavus were isolated based on macro- and microscopic morphological characteristics. All characteristics (i.e. spore arrangement, spore morphology, and pattern and colour of the fungal colony) of the targeted A. flavus strains were identified using a classic species description reference (Klich, 2002).
The *A. flavus* colonies were identified genetically, as described in our previous study (Al-Shuhaib *et al.*, 2018).

**Extraction of Aflatoxins**

All 109 isolates of *A. flavus* were grown separately on 100 mL of potato dextrose liquid culture medium at 35ºC for 18 days. Then, 50 mL of the extracellular mycelial growth was mixed with an equal volume of chloroform and incubated on a rotary shaker for 4 hours. After the toxins dissolved in the solvent, the aqueous layer was discarded, and the remaining fraction was stored at 50ºC until all of the chloroform evaporated. The leftover precipitate was dissolved thoroughly with 1 mL of methanol.

**HPLC**

HPLC experiments were performed at the Department of Clinical Laboratory Analysis, College of Pharmacy, University of Babylon. Before injection, the samples were filtered through 0.45-mm filters (Millipore Corporation, Bedford, MA, USA). Then, 20 µL of the supernatant was injected into an HPLC system equipped with a photodiode array detector, which provides high sensitivity, baseline stability, and analytical reliability. The system control and data acquisition were performed using LabSolutions LC WorkStation software (Ver.5.51; Shimadzu, Kyoto, Japan). The analyses were conducted with a SUPELCOSIL™ LC-18 HPLC column (LxID: 25 cm×4.6 mm, Particle size: 5 µm; Cat. No. 58298; Supelco, Bellefonte, PA, USA) at 40ºC using acetonitrile, methanol, and water (20, 20, and 60%, respectively) as a mobile phase with a flow rate of 1.5 mL min⁻¹ for 15 minutes, following the manufacturer’s instructions. To identify the AFs, the observed retention time and UV absorbance spectra of sample peaks were compared and matched to those obtained from an AF mix reference standard (Cat. No. 46304-U; Supelco). Quantitation of AFs was conducted by comparing the peak area of the target peaks (at 365 nm) to the standard curve generated using several dilutions of the AF mix reference standard.

**RESULTS AND DISCUSSION**

This detailed study on AF production by 109 *A. flavus* isolates revealed that 71% (*n* = 77) of the total isolates were aflatoxigenic (Figure 1 and Table 1). The low number (*n* = 32; 29%) of non-aflatoxigenic *A. flavus* clearly demonstrates the importance of screening for rice-infecting *A. flavus* strains and the impact of AFs in general (and AFB₁ in particular) on human and animal health. The analyzed strains presented a variety of synthesized AF combinations and concentrations. Vaamonde *et al.* (2003) observed similar differences in the ability of *A. flavus* strains to produce AFs in various crops. Notably, the isolates included mono-, bi-, and tri-aflatoxigenic strains, but no observed isolate appeared capable of producing all four AFs together. Although a previous report indicated the concomitant absence of AFB₂, AFG₁, and AFG₂ (Dhanasekaran *et al.*, 2011), the rice-contaminating *A. flavus* isolates in the present study produced four types of AFs (Table 1). These results contrast with those of previous studies indicating the ability of *A. flavus* strains to produce only AFB₁ and AFB₂ (Ehrlich *et al.*, 2004; Frisvad *et al.*, 2005). However, the present results were consistent with the observed production of AFG₁, AFG₂, AFB₁, and AFB₂ by *A. parasiticus* strains (González-Salgado *et al.*, 2008). It has been reported that a high percentage of *A. flavus* strains synthesize AFG₁, and a minor group also accumulates AFG₂ (Karaaslan and Arslangray, 2015). Certain *A. flavus* strains have been shown to produce G-type AFs (Pildain *et al.*, 2008). Another study reported that AFB- and AFG-producing *A. flavus* strains belong to group II of the same species, which differ from the main group I strains that produce only AFBs.
(Geiser et al., 2000). In the present study, although most of the strains were found to produce the four main AFs, AFB$_1$ concentration and prevalence were consistently higher than those of the other three AFs (Table 1). In fact, AFB$_1$ accounted for more than half of the total AF content (Figure 1-b). The HPLC results showed that AFB$_1$, AFG$_2$, AFG$_1$, and AFB$_2$ were produced by 49, 45, 16, and 14 aflatoxigenic isolates, respectively, out of the 109 A. flavus strains analyzed (Figure 1-c). The results clearly demonstrated that AFB$_1$ had the highest concentration in many A. flavus strains.

Figure 1. The pattern of aflatoxicogenicity in 109 Aspergillus flavus isolates infecting rice grains: (A) The numbers of mono-, bi-, tri-, and non-aflatoxigenic isolates; (B) Mean concentration of Aflatoxins (AFs) observed in Aspergillus flavus isolates infecting rice grains; (C) The prevalence of AF types observed in the Aspergillus flavus isolates.
Table 1. The concentrations of aflatoxins $B_1$, $B_2$, $G_1$, and $G_2$ (µg mL$^{-1}$) in each *Aspergillus flavus* strain.*

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*No: Refers to the strain number included in the present study. B1: AFB$_1$ is in pink; G1: AFG$_1$ is in grey; B2: AFB$_2$ is in green; G2: AFG$_2$ is in cyan. The mean±SD of the toxins: B1: 728.20±2750.61; B2: 102.05±185.15; G1: 36.52±59.7; G2: 17.61±27.96.*
strains (Table 1), with an average concentration of 3,561.9 µg kg⁻¹. This AF₁ level was considerably higher than the allowable limit (≤30 µg kg⁻¹) (Nguyen et al., 2007). Our results are consistent with studies indicating that AF₁ is the most prevalent AF contaminant and has the highest concentration among rice-infecting A. flavus isolates (Lai et al., 2015; Reddy et al., 2011; Sun et al., 2017). The high prevalence and concentration of AF₁ makes the daily consumption of stored rice grains potentially dangerous. One limitation of this study was a lack of data on the storage duration of the collected rice grains. Therefore, further large-scale or national-level studies are required to avoid foodborne intoxication. Yazdanpanah et al. (2013) also recommended regular screening for AF₁-contaminated rice. Such screening is critical because the consumption of a high quantity of AF₁ is toxic to the liver; the cyclic nucleotide phosphodiesterase activity in the brain, liver, heart and kidney tissues can be inhibited by AF₁, resulting in severe impairment of the metabolism of proteins, carbohydrates, and lipids in the liver (Bonsi et al., 1999). Chronic intake of such toxins could act synergistically with other factors, such as the hepatitis B virus, to promote liver cancer (Chawanthayatham et al., 2017). In this study, AF₁ had the second highest concentration among the 109 isolates (1,632 µg kg⁻¹). Previous studies have shown that rice is susceptible to AF₁ and AF₂ accumulation (Lai et al., 2015; Reddy et al., 2011). Both the present observations and previous results indicate that rice grains are a major source of AF contamination. These findings reaffirm that the risk of AF poisoning in humans via rice contaminated by toxigenic A. flavus strains cannot be eliminated unless proper measures are taken. Consumption of rice is high in many countries; therefore, monitoring the concentrations of AF₁ in stored rice grains is strongly recommended as a preventative measure. The occurrence of AFs in most samples analyzed herein indicates that there is a need for regular, national-level programs to monitor the concentrations of AFs in stored rice grains. The concentration of AFs should be reduced by adopting proper storage practices, such as Good Agricultural Practices (GAP) and Good Handling Practices (GHP) in Iraq.

CONCLUSIONS

AF contamination by rice-infecting A. flavus strains is a serious concern. AF₁ was the most prevalent of the four AFs analyzed in A. flavus strains isolated from rice grains. The results clearly indicated that the conditions and methods of rice storage used in Iraq were favourable for the growth of A. flavus and AF contamination. Therefore, future studies aiming to elucidate any possible etiological influence of AFs on the incidence of liver cancer in Iraq are strongly recommended. Strict regulations should also be devised and implemented to prevent further synthesis of AFs on rice grains.

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شیوع بالای افلاتاکسین ۱ در بی. فلوروسیس اسپرگیللاس flavus در آلوهده کنده دانه برنج درتابار

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چکیده

یکی از ریختگی‌های‌آلوده کنده دانه برنج به‌خوبی شناخته شده است. هلدین این پژوهش بررسی پانسل پیوندن A. flavus، این AFG1، AFG2، AFB1، AFB2، FS گیری‌های‌ آلوده‌کنده در سلامت و بیماری‌های مختلف در عراق و در طی سال‌های ۲۰۱۵ و ۲۰۱۶ یکی از آلوده‌کنده‌های A. flavus به‌خوبی شناخته شده است. افلاتاکسین‌ها عصاره‌گیری شد و مقدار شان با استفاده از هم‌مردانه‌گرافی می‌باشد (photodiode array detector) تعمین می‌شود. نتایج آنلیست داشته که ۹۲% از آلوده‌کنده‌های A. flavus که ظرف‌توسینف در عراق در حال حاضر به‌خوبی شناخته شده است. کنده‌های آلودگی فلوروسیس اسپرگیللاس flavus در حال حاضر به‌خوبی شناخته شده است. در حال حاضر، فلوروسیس اسپرگیللاس flavus در عراق در حال حاضر به‌خوبی شناخته شده است. می‌تواند به‌خوبی شناخته شده است. می‌تواند به‌خوبی شناخته شده است.