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High Prevalence of Aflatoxin B_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_1, AFB_2, AFG_1, and AFG_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_1, AFG_2, AFG_1, and AFB_2 were produced by 49 (45%), 44 (40%), 20 (18%), and 17 (16%) strains, respectively, at various concentrations. The concentration of AFB_1 was the highest among the *A. flavus* strains, with a mean value of 3,561.9 µg kg^{-1}. In conclusion, the most abundant AF synthesized by the rice-infecting *A. flavus* strains was AFB_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: Aflatoxigenic, 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 aflatoxigenic *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*

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al., 2007). At present, about 20 AFs have been identified, but those most frequently found in foods like cereals, rice, and corn are AFB_1, AFB_2, AFG_1, and AFG_2 (Li *et al.,* 2014), of which AFB_1 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 AFB_1 (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).

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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^{TM} LC-18 HPLC column (L×ID: 25 cm×4.6 mm, Particle size: 5 µm; Cat. No. 58298; Supelco, Bellefonte, PA, USA) at 40°C 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 AFB_1 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 AFB_2, AFG_1, and AFG_2 (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 AFB_1 and AFB_2 (Ehrlich *et al.*, 2004; Frisvad *et al.*, 2005). However, the present results were consistent with the observed production of AFG_1, AFG_2, AFB_1, and AFB_2 by *A. parasiticus* strains (González-Salgado *et al.*, 2008). It has been reported that a high percentage of *A. flavus* strains synthesize AFG_1, and a minor group also accumulates AFG_2 (Karaaslan and Arslanğray, 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

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(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*

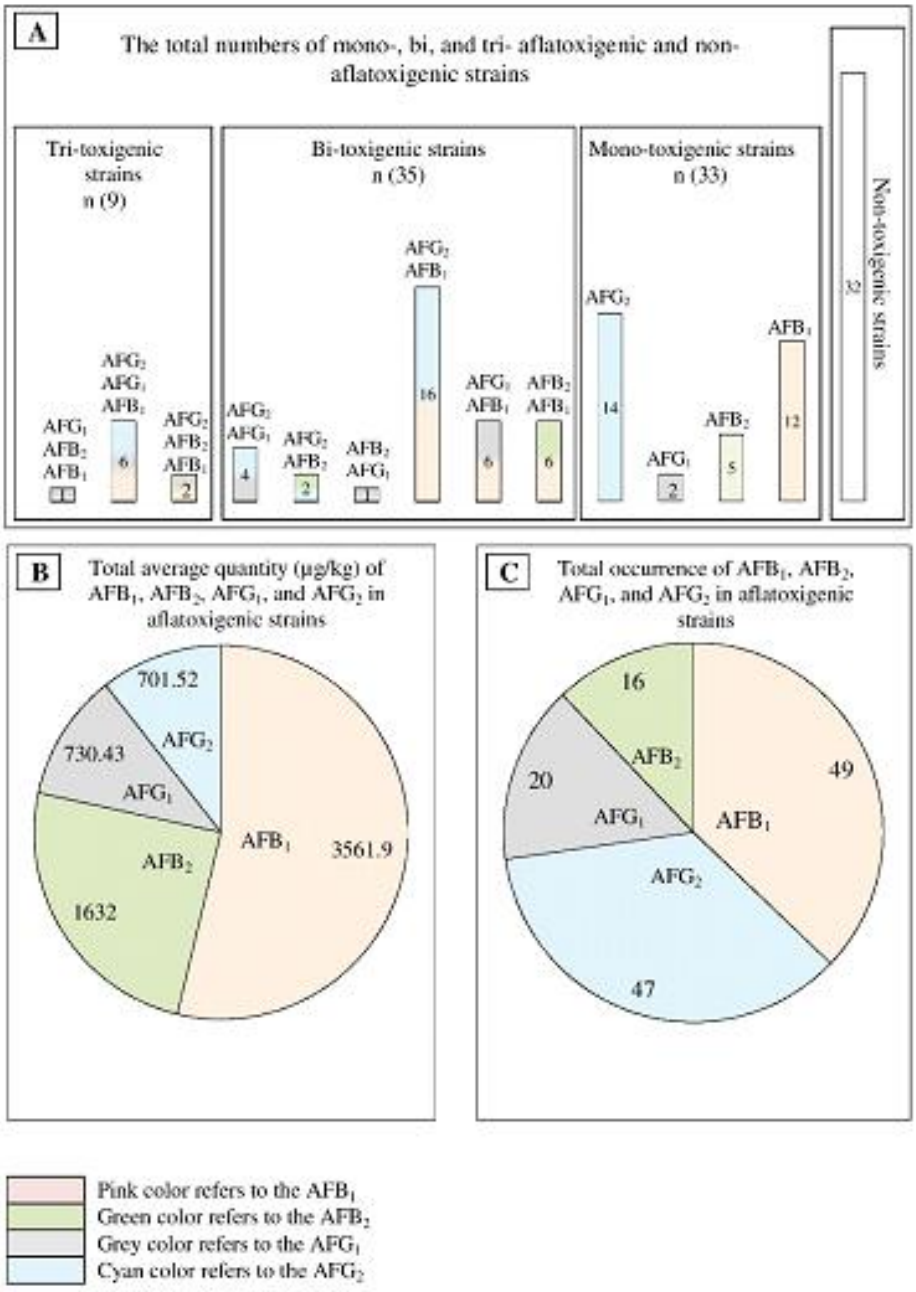

Figure 1. The pattern of aflatoxigenicity in 109 *Aspergillus flavus* isolates infecting rice grains: **(A)** The numbers of mono-, bi-, tri-, and non-aflatoxigenic isolates; **(B)** Mean concentration of Aflatoxins (AFs) observed in *Aspergillus flavus* isolates infecting rice grains, **(C)** The prevalence of AF types observed in the *Aspergillus flavus* isolates.

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Table 1. The concentrations of aflatoxins B_1, B_2, G_1, and G_2 (µg mL^{-1}) in each *Aspergillus flavus* strain.[a]

No.	B1	B2	G1	G2	No.	B1	B2	G1	G2	No.	B1	B2	G1	G2	No.	B1	B2	G1	G2
1	244.04	-	8.07	26.65	29	90.84	-	-	3.77	57	-	11.67	-	2.80	85	412.54	-	10.72	4.41
2	245.76	-	-	19.24	30	-	-	-	24.09	58	-	17.62	-	-	86	82.77	-	-	-
3	12.80	-	-	9.29	31	-	24.43	-	11.01	59	562.88	-	-	-	87	413.51	-	-	-
4	-	-	-	0.67	32	-	-	-	11.63	60	-	-	-	-	88	687.65	-	-	-
5	-	-	14.09	4.04	33	-	-	-	-	61	903.03	-	116.47	-	89	292.61	-	-	-
6	129.03	-	-	6.75	34	8.18	-	-	-	62	-	-	9.91	6.00	90	18483.5	148.52	-	-
7	-	-	-	0.67	35	12.00	-	-	-	63	33.31	-	18.56	3.06	91	263.48	45.69	-	1.12
8	38.18	-	-	0.67	36	-	-	-	40.71	64	-	-	-	-	92	921.93	-	-	48.18
9	5.64	-	-	6.45	37	-	-	-	-	65	-	-	-	90.95	93	715.64	-	245.46	-
10	-	-	-	5.57	38	-	-	-	-	66	-	57.34	-	-	94	180.15	-	-	-
11	-	-	6.73	2.39	39	-	739.20	-	-	67	-	-	-	-	95	473.30	-	-	8.34
12	-	-	-	2.52	40	-	-	-	-	68	-	-	-	-	96	569.11	-	-	18.08
13	47.47	-	-	7.35	41	-	-	-	-	69	-	-	-	-	97	94.88	-	-	4.13
14	10.19	-	-	8.94	42	-	-	-	-	70	292.36	39.62	-	-	98	124.00	-	16.55	-
15	7.14	-	-	2.82	43	-	7.70	-	-	71	-	-	-	-	99	30.31	-	-	-
16	-	-	-	5.25	44	-	-	-	-	72	246.63	76.65	-	-	100	21.79	-	60.52	16.81
17	-	-	-	25.94	45	-	-	-	-	73		-	6.17	2.43	101	-	-	-	-
18	-	-	-	-	46	-	-	-	-	74	9.56	-	-	-	102	-	274.15	-	11.03
19	29.79	-	-	-	47	-	-	-	-	75	695.31	-	-	71.50	103	-	-	-	-
20	-	-	-	-	48	-	-	-	-	76	-	-	-	-	104	-	-	7.60	-
21	-	-	-	-	49	-	-	-	-	77	16.03	-	-	78.87	105	-	-	-	23.68
22	-	-	-	-	50	-	-	-	-	78	34.45	-	11.79	147.16	106	-	-	-	-
23	14.85	12.85	-	-	51	-	-	24.45	-	79	-	-	-	24.26	107	1321.9	-	-	-
24	37.70	-	9.08	1.96	52	-	-		-	80	3.53	-	-	-	108	238.82	-	-	-
25	-	-	-	3.42	53	-	4.78	6.79	-	81	50.67	-	-	8.36	109	22.93	150.58	118.78	-
26	-	-	-	-	54	3.85	5.84	-	-	82	31.47	-	6.60	-					
27	-	-	-	-	55	6389.1	15.44	-	6.80	83	-	-	-	4.41					
28	-	-	7.15	3.29	56	-	-	-	-	84	125.29	-	24.94	10.28					

[a] 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 AFB_1 level was considerably higher than the allowable limit (~30 µg kg^{-1}) (Nguyen *et al.*, 2007). Our results are consistent with studies indicating that AFB_1 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 AFB_1 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 AFB_1-infected rice. Such screening is critical because the consumption of a high quantity of AFB_1 is toxic to the liver; the cyclic nucleotide phosphodiesterase activity in the brain, liver, heart and kidney tissues can be inhibited by AFB_1, 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, AFB_2 had the second highest concentration among the 109 isolates (1,632 µg kg^{-1}). Previous studies have shown that rice is susceptible to AFB_1 and AFB_2 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 AFB_1 in stored rice grains is strongly recommended as a preventive 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. AFB_1 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 B_1 در *Aspergillus flavus* آلوده کننده دانه برنج درانبار

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چکیده

یکی از ریزجانداران آلوده کننده برنج که به خوبی شناخته شده، *Aspergillus flavus* است که متابولیت های سمی به نام افلاتاکسین (AFs) تولید می کند. هدف این پژوهش بررسی پتانسیل بیوسنتز همزمان چهارAFs AFB1 (، AFB2،AFG1 و AFG2) در سویه های آلوده کننده *A. flavus* بود. شیوع یا فراوانی افلاتاکسین در ۱۰۹ سویه *A. flavus* که از دانه های برنج هندی انبار شده در ۳۰۰ مکان مختلف واقع در منطقه فرات میانی در عراق و در طی سالهای ۲۰۱۵ و ۲۰۱۶ جمع آوری شده بود بررسی شد. افلاتاکسین ها عصاره گیری شد و مقدارشان با استفاده همزمان از کروماتوگرافی مایع با کارایی بالا (HPLC) مجهز به ردیاب آرایه فتو دیود (photodiode array detector) تعیین گردید. نتایج آشکار ساخت که ۲۹٪ (n=32) از سویه ها غیر-افلاتاکسیونی (non-aflatoxigenic) و مابقی یعنی ۷۱٪ (n=77) به طور تایید شده ای افلاتاکسینی بودند که توانایی های متغییری برای تولید ,mono-, bi-و tri-Afs داشتند. همچنینAFB_1،AFB_2 AFG_1, AFG_2 به ترتیب توسط ۴۹ (۴۵٪)، ۴۴(۴۰٪)، ۲۰(۱۸٪)، و ۱۷ (۱۶٪) ازسویه ها در غلظت های مختلف تولید شد. در میان همه سویه های *A. flavus* ،غلظتAFB_1 با میانگین3,561.9 µg/kg ازهمه بیشتر بود. نتیجه اینکه ، AFB_1 فراوان ترین افلاتاکسین تولید شده سویه *A. flavus* بود که آلوده کننده برنج است. آلودگی با افلاتاکسین ها همچنان خطرات بالقوه سلامتی را برای حیوانات و انسانها ایجاد می کند. این نتایج به روشنی نشان می دهد که شرایط نامناسب انبارداری برنج در عراق برای رشد *A. flavus* و آلوده کرن با افلاتاکسین مساعد است. بنا براین مطالعاتی در سطح ملی برای جلوگیری از مسمومیت های ناشی از غذا واجب است. مقررات سختی باید وضع و اجرا گردد تا از تولید افلاتاکسین روی دانه های برنج جلوگیری شود .

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