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Full Length Research Paper

Evaluation of the correlation between Aflatoxin B1 production and *Aspergillus* contamination in rice bran from northern Iran

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Aflatoxins are potent mycotoxins, which are produced by toxigenic *Aspergillus* species including *Aspergillus flavus* and *Aspergillus parasiticus*. The goals of this study were to characterize *Aspergillus* fungus isolated from rice bran and to determine the correlation between Aflatoxin B_1 (AFB_1) production and *Aspergillus* contamination in the samples. Thirty rice bran samples were collected from different regions of Mazandaran province, northern Iran. Samples containing 15 specimens were conserved for one year in the storage and the other one was not subjected to storage. Mycological analysis of rice bran was performed and AFB_1 was purified from the samples and quantified by high performance liquid chromatography (HPLC) method with fluorimetry detection. The results of this study showed that almost all collected samples contained AFB_1. The averages of AFB_1 in new and old rice bran samples were found to be 5.07 and 6.81 µg/kg, respectively. No significant difference was obtained between AFB_1 value in new and old samples. In addition, the correlation between culture results and aflatoxin production were significantly observed only in old samples ($P<0.05$). The most frequent isolated *Aspergillus* species were *Aspergillus terreus*, *A.flavus*, *Aspergillus niger*, *A. parasiticus* and *Aspergillus fumigatus*. In conclusion, these results confirmed that rice bran could be contaminated with AFB_1 and toxigenic *Aspergillus* species and human beings are in danger of been affected by contaminated rice products.

Key words: Aflatoxin B_1, rice bran, toxigenic *Aspergillus* species.

INTRODUCTION

Rice (*Oryzae sativa L.*) is a very important foodstuff for millions of people in Iran. It is the dominant grain for half of the world population and provides 20% of the world's dietary energy supply, with wheat and maize supplying 19 and 5%, respectively (FAO, 2004). Fungal contamination in cereal grains, which can occur at the farm or at the storage site, affects the yield, quality and nutritional value of the products (Aran and Eke, 1987). Continuous and heavy rainfalls in some regions of different countries moisturize the crop and make panicles more prone to invasion by *Aspergillus* species (Reddy et al., 2005).

Mycotoxins potentially present in mouldy rice include aflatoxins, Ochratoxin A and Fusariotoxins (Miraglia and Brera, 2000). The Food and Agriculture Organization (FAO) estimates that at least 25% of the world cereal production is contaminated with mycotoxins (Dowling, 1997). Aflatoxins are toxic secondary metabolites produced by some species of *Aspergilli,* especially *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*. Aflatoxin B_1 (AFB_1) is the most potent hepatocarcinogen known in mammals. It presents hepatotoxic, teratogenic and mutagenic properties, causing damage such as toxic hepatitis, hemorrhage, edema, immunosuppression and hepatic carcinoma

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(Speijers and Speijers, 2004; Reddy et al., 2005).

A number of surveys and monitoring programs have been carried out in several countries attempting to obtain a general pattern of aflatoxin contamination in rice (Reddy et al., 2008). Different studies have been conducted concerning the natural occurrence of AFB_1 in rice in Sri Lanka (Breckenridge et al., 1986), China (Liu et al., 2006) and India (Mangala et al., 2006). To date, there have been no studies on detection of *Aspergillus* species and AFB_1 in rice in Iran. Iran is one of the largest producers of rice in the Middle East; it has critical importance in the world rice trade. The purpose of this study was to determine the correlation between AFB_1 and *Aspergillus* contamination in the new and old rice bran samples in northern Iran. To quantify the amounts of AFB_1 in rice bran, a simultaneous analytical method was developed by using fluorimetry detection system.

MATERIALS AND METHODS

Rice bran collection

A total of 30 rice bran samples were randomly collected from 30 locations in Mazandaran province in northern Iran between August and September 2009. Fifteen samples were stored for one year while the other ones were the fresh samples. To avoid sampling error due to the highly heterogeneous nature of fungal distribution, each 1 kg composite sample collected from one storehouse was a mixture of 4 sub-samples (25 g each).

Isolation of Aspergillus species from rice bran samples

The samples were cultured on Sabouraud glucose agar and Czapek-dox agar (Diba et al., 2007). The plates were incubated at 28°C temperature for 6 days and then the fungal species of each colony was identified (Klich, 2002). In this study, all general chemical materials were procured from Merck Co., Darmstadt, Germany.

Preparation of standard solutions

Standard solution of AFB_1 (St Louis, MO, USA) was prepared by dissolving 10 mg of AFB_1 in 1 ml of methanol. The concentration of the AFB_1 stock solution was determined by measuring the UV absorbance at 360 nm (IARC, 1982). Also underivatized AFB_1 was separated by HPLC and detected by spectrofluorimetry after post-column derivatization in the Kobra cell, where it was converted in to the 9, 10- dibromo derivative.

Extraction and purification of AFB_1 from rice bran samples by HPLC

For extraction of toxin, 20 g of rice bran was mixed with 100 ml of 4% acetonitrile aqueous solution of potassium chloride (9:1). Extraction was followed by shaking for 20 min and filtered through Whatman No.4 filter paper under vacuum condition.

For purification, 100 ml of n-hexane were added to the filtrate and shaken for 10 min. After separating, the upper phase (n-hexane) was discarded. To the lower phase, 50 ml deionized water and 50 ml chloroform were added and this solution was shaken for 10 min. After separation, the lower phase was collected and the upper phase was re-extracted twice with 25 ml of chloroform by using the above conditions. Then the chloroform was evaporator in a 40°C water bath at low speed. Methanol at the rate of 2 ml was added and the solution was sonicated, filtered through a 0.45 µl filter and finally evaporated to dryness under nitrogen. For HPLC analysis system, 500 µl of methanol were added. A Shimadzu LC-10, equipped with an injector 20 µl loop, a C18 spherisorb column and a fluorescence detector were used. Excitation and emission wave lengths were 365 and 440 nm, respectively. The system was run isocratically with mobile phase acetonitrile/propanol-2-o1 and flow rate of 0.5 ml/min (Hansen, 1993).

Data analysis

T-student and chi-square tests were used to asses of the results. A p value less than 0.05 was statistically considered significant.

RESULTS

As shown in Table 1, all samples showed AFB_1 positive. Based on statistical analysis, the mean values of AFB_1 in the old and new samples were calculated as 6.81 and 5.07 µg/kg, respectively. Although the amount of AFB_1 in old samples was higher than new ones, there was no significant linear relationship between the amount of AFB_1 and storage period.

The *Aspergillus* species recovered from the samples were given in Figure 1. The frequency of *Aspergillus* species isolated from old and new samples were found to be 41 and 37%, respectively. Contamination of *A. flavus* and *A. terreus* was dominated in all the storage conditions. Five different *Aspergillus* species were identified, which included *A. flavus*, *A. terreus*, *A. fumigatus*, *A. niger* and *A. parasiticus* (Figure 2). The correlation between fungal culture and AFB_1 production was only significant in old samples ($P<0.05$).

DISCUSSION

Rice is an aquatic plant and is usually harvested at much higher moisture levels (30 to 50%). Mycotoxin-producing fungi may contaminate grains and produce high quantities of these toxins during storage of this product (Park et al., 2005). In our study, rice bran samples collected from different storage conditions showed infection by *Aspergilli*. The contamination of toxigenic *Aspergillus* species such as *A. flavus* was dominated in these samples. These results confirm the earlier observations of Reddy et al. (2004) who had also reported *Aspergillus* species as one of the most predominant fungus in rice samples. Similarly, the others have reported *Aspergillus* species as one of the most predominant fungi in grain from flood-affected paddy fields in India (Begum and Samajpati, 2000; Reddy et al., 2009). Sundaram et al. (1988) reported the presence of *Aspergillus*, *Penicillium* and *Fusarium* species from 150

Table 1. Comparative amounts of AFB_1 in different new and old samples of rice bran.

AFBI	Valid		No. (%)		Cumulative percent	
Sample	**New**	**Old**	**New**	**Old**	**New**	**Old**
1	1.25	1.2	1(6.7)	1(6.7)	6.7	6.7
2	1.48	1.25	1(6.7)	1(6.7)	13.3	13.3
3	1.57	2.1	1(6.7)	*	20	*
4	1.58	2.1	1(6.7)	2(13.3)	26.7	26.7
5	12.8	3.32	1(6.7)	1(6.7)	33.3	33.3
6	2.81	4.48	1(6.7)	1(6.7)	40	40
7	3.2	5.75	1(6.7)	1(6.7)	46.7	46.7
8	3.4	6.48	1(6.7)	1(6.7)	53.3	53.3
9	5.32	7.4	1(6.7)	1(6.7)	60	60
10	5.71	9.32	1(6.7)	*	66.7	*
11	6.32	9.32	1(6.7)	2(13.3)	73.3	73.3
12	6.33	9.93	1(6.7)	1(6.7)	80	80
13	6.75	11.87	1(6.7)	1(6.7)	86.7	86.7
14	7.6	13.1	1(6.7)	1(6.7)	93.3	93.3
15	9.8	14.5	1(6.7)	1(6.7)	100	100

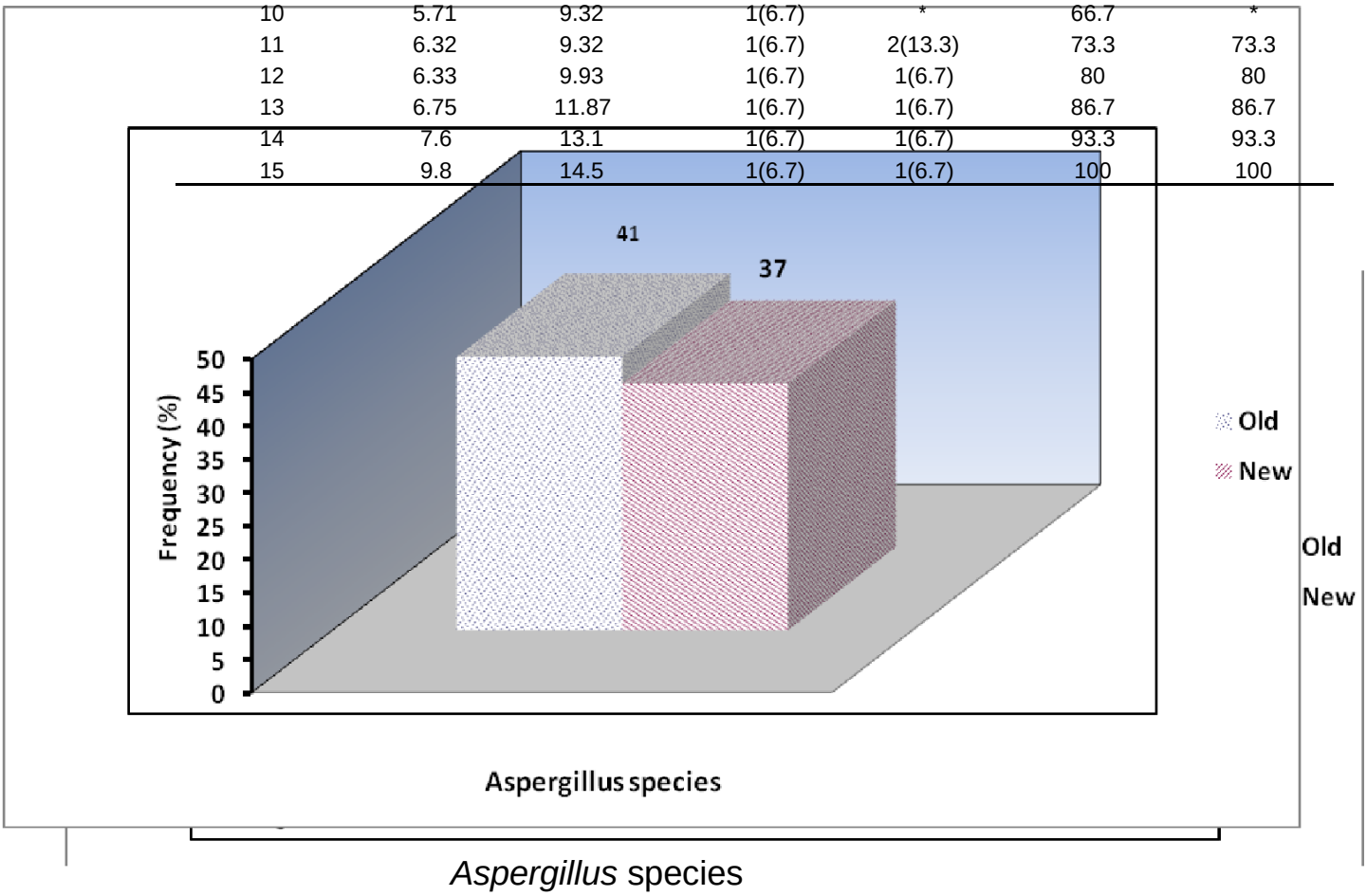

Figure 1. The frequency of *Aspergillus* species in new and old samples of rice bran (%).

stored rice samples. *A. flavus* and *A. terreus* were dominated in their presence and they were high in the samples stored for one year. Chary and Reddy (1987) reported 14 fungal species representing 6 genera that were isolated from rice. The predominant *Aspergillus* species were *A. flavus*, *A. fumigatus* and *A. nidulans.* Reddy et al. (2009) showed that *A. flavus* and *A. niger* contaminations were dominant in milled rice samples.

Previous studies demonstrated the effects of drought and temperature on AFB_1 production and *A. flavus* contamination (Cole et al., 1989; Dowd, 1998; Moreno and Kang, 1999). Mazandaran province selected in this study is located in northern Iran. It covers with moderate weather conditions; relatively warm and humid all year around. It was proven that climate shift in this region causes the growth of aflatoxin producing fungi (Payne et

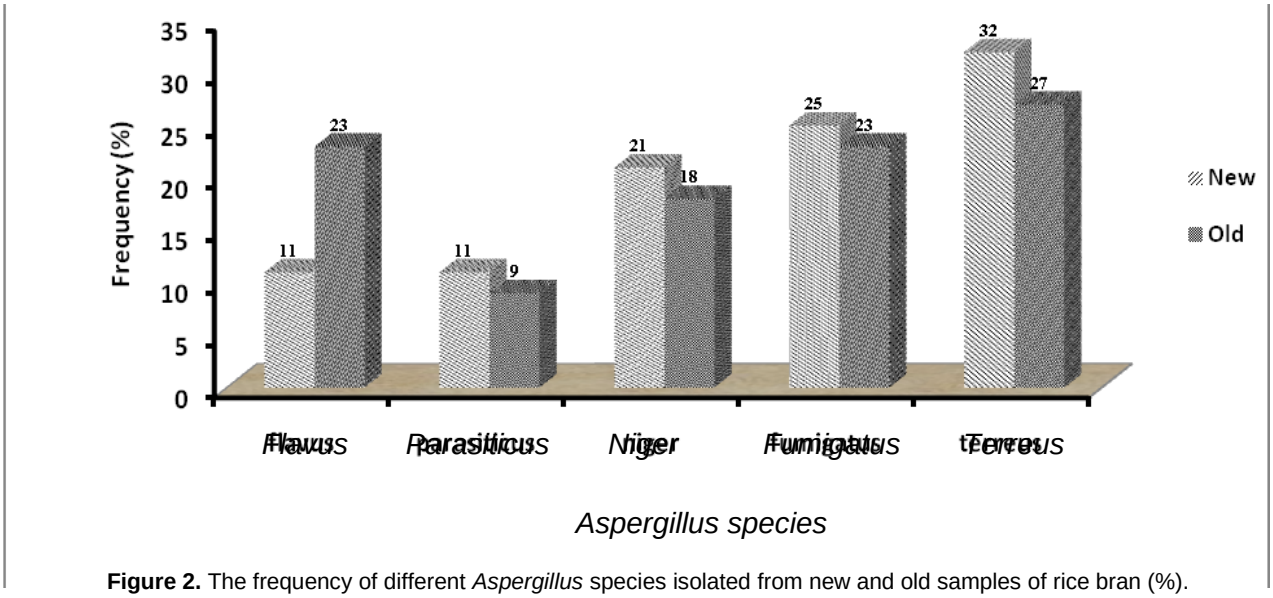

Figure 2. The frequency of different *Aspergillus* species isolated from new and old samples of rice bran (%).

al., 1988). This includes changes in the quantity of aflatoxin-producers in the environment and alteration to fungal community structure. Our study demonstrated the presence of AFB_1 in the samples. In this study, 41% of old samples and 37% of new samples were found to contain AFB_1 levels ranging from 1.25 to 12.8 and 1.2 to 14.5 µg/kg, respectively. In the United Arab Emirates, AFB_1 was detected in 160 (64%) long grain rice samples and 81 (32%) short grain rice samples at levels ranging from 1.2 to 16.5µg/kg (Osman et al., 1999). Sales and Yoshizawa (2005) reported that the incidence of AFB_1 in rice ranged from 0.025 to 11.0 µg/kg in the Philippines. In another study, in which AFB_1 was estimated for 1200 samples by ELISA, 67.8% of the samples were positive to AFB_1 (Reddy et al., 2009). Toteja et al. (2006) examined parboiled rice collected from India and found 38.5% of the samples to be positive for AFB_1. More recently, 9% of rice samples in Ecuador were shown to be contaminated with aflatoxins with a range of 6.8 to 40 µg/kg (Mühlemann et al., 1997). Bandara et al. (1991) analyzed 597 rice samples and AFB_1 was detected in 72 (12%). In our study, the percentage of samples positive for AFB_1 was similar to that reported by Sales and Yoshizawa (2005) and Osman et al. (1999). The AFB_1 contamination was detected in 37 samples of milled rice grains and found that 92% of the samples showed positive to AFB_1. Prasad et al. (1987) tested 56 samples of stored rice and 12 were positive for aflatoxin. Levels of aflatoxins ranged from 184 to 2830 mg/kg.

The AFB_1 value was higher than the minimum standard institute regulated level in this study. Since rice bran is main diet of the local individuals and consumed in large quantities, aflatoxin contamination could be a serious even at low levels (Paranagama et al., 2003). According to our findings, the AFB_1 amounts in the samples stored for one year was higher than the non-stored ones. In addition, *A. flavus* was isolated from old samples rather than new samples as well. *A. flavus*, which produces only AFB_1, were present on rice bran. That can be reason for the increasing AFB_1 in the old samples.

AFB_1 contamination can be divided into two distinct phases with infection of the developing crop in the first phase and increases in contamination after maturation in the second phase (Cotty, 2001). During the first phase of contamination wounding of the developing crop by birds, insects, mechanically or the stress of hot dry conditions results in significant infections (Dowd, 1998; Guo et al., 2003). The second phase occurs when the mature crop is exposed to warm and humid conditions either in the field or during transportation and specially storage. Of course, compositions of fungal communities set up during the first phase greatly influence the second phase (Cotty, 2001). Therefore, the management of storage is very important and improvement in storage conditions for preventing the spoilage and reducing the AFB_1 contamination is recommended (Sundaram et al., 1988; Li, 1997).

Conclusion

This study demonstrated that feedstuffs in northern Iran could be contaminated with AFB_1. The risk of human exposure to AFB_1 in contaminated grains is an important public health issue.

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