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Grain-Borne Mycoflora and Fumonisin B1 From Fresh-Harvested and Stored Rice in Northern Iran

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Background: Fumonisins B1 (FB1) is the main member of the family of fumonisins produced by several *Fusarium* species in cereals, especially rice.

Objectives: The purpose of this study was to analyze mycoflora and FB1 contamination of fresh and stored rice grains.

Materials and Methods: One-hundred and fifty different fresh and stored rice samples were collected from 30 different zones of the Mazandaran province, Iran between August 2010 and November 2011. After sterilization, the grains were cultured on potato dextrose agar (PDA) containing chloramphenicol (100 mg/L) at 27°C for 7-10 days. All *Fusarium* isolates were sub-cultured on PDA, SpezielleNährstoffarmer agar (SNA) and carnation leaf agar (CLA). FB1 was extracted with acetonitrile: water (50: 50, v/v) solution and detected by high-performance liquid chromatography (HPLC) analysis using a fluorescence detector (excitation: 229 nm; emission: 442 nm).

Results: Mycoflora profiles of fresh and stored rice grains showed that *Aspergillus* species (37.3%, 40.7%) were the predominant fungal agents, followed by *Fusarium* (21.6%, 16.2%), *Mucor* (19.6%, 16.7%) and *Rhizopus* (9.8%, 11.1%), respectively. In HPLC analysis, most of the rice samples (96.7%) collected were found to be positive for FB1 with mean levels ranging from not detected to 56.2 mg/kg for fresh samples and from 4.3 to 42.8 mg/kg for stored ones. FB1 levels varied from one zone to another and throughout the storage time, showing a decreasing trend in most zones.

Conclusions: Rice samples with a high prevalence of diverse species of toxigenic fungi, in particular *Aspergillus* and *Fusarium* species, and high levels of FB1 in many samples indicate the need for proper surveillance and monitoring exclusively for the prevention of fungi and FB1 in rice produced in Mazandaran province before it reaches the consumer.

Keywords: Fusarium; Fumonisin B1; Chromatography, High Pressure Liquid; Rice; Iran

1. Background

Mycotoxin contamination in cereals is a potential risk to human and animal health. Among several hundreds of mycotoxins, fumonisins are *Fusarium* toxins and are among the most important mycotoxins regarding food safety (1). The presence of different species of *Fusarium*, in particular *F. verticillioides*, (Sacc.) Nirenberg (*F. moniliforme* Sheldon), *F. proliferatum, F. nygami, F. anthophilum, F. napiforme, F. dlamini*and *F. globosum*, in food substrates is relevant not only because the potential role of such fungi in these products decays during storage and marketing but also because of the fact that some strains can produce mycotoxins (2).

Currently, 28 structural fumonisin analogs are known, and the most abundant analog in nature is fumonisin B1 (FB1), followed by FB2 and FB3 (3). FB1 has been implicated in animal disorders such as leukoencephalomalacia in horses, pulmonary oedema syndrome in pigs and nephrotoxicity, hepatotoxicity and hepatocellular carcinogenicity in rats (4). Furthermore, an association between high rates of human esophageal cancer and high concentration of fumonisin in cereals has been reported in different countries (5). In 2001, the Joint FAO /WHO Expert Committee on Food Additives and Contaminants (JECFA) established a Provisional Maximum Tolerable Daily Intake (PMTDI) for fumonisins (the sum of FB1, FB2 and FB3) of 2.0 μg/kg of body weight per day (6) and in 2002, the International Agency for Research on Cancer (IARC) determined that the FB1 derived from *F. verticillioides* belongs to Group 2B, i.e. a possible human carcinogen (7).

Fumonisins are mainly found as natural contaminants of corn and corn-based foods (8). However, there is evi-

Implication for health policy/practice/research/medical education:
The public health implications of the presence of fumonisin B1 and many mycotoxigenic fungi found in rice in Iran causes concern particularly if these compounds and fungi are synergistic with each other. This makes regulation of mycotoxins in our foods and foodstuffs, an imperative.

dence that they can occur in other crops such as sorghum (9), bean (10), wheat, barley and soybean (11) and black tea and medicinal plants (12). Rice (Oryza sativa) is considered as the most important staple food for the human population Worldwide, in particular in the Middle East. This grain is the second highest worldwide production after corn (13). Although contamination of rice with fumonisins has been reported in the United States (14) and it has been studied extensively in the European Union (15), little information has come from Asia (16). As the legal limits vary significantly both from country to country and by mycotoxin type and matrix; the determination methods need to provide accurate and reproducible results both within and between laboratories.

2. Objectives

The natural occurrence of FBI in rice and rice-based foods from northern Iran had not been studied to date, so the present study using high-performance liquid chromatography (HPLC) analysis coupled to a fluorescence detector determined the FB1 content of *Fusarium*-infected fresh and stored rice grains and described its mycoflora profile.

3. Materials and Methods

3.1. Rice Samples

One-hundred and fifty different fresh (No. 75) and stored (No. 75; contamination lasting more than 12 months) rice samples intended for human consumption were collected from 30 different zones (5 samples from each zone) of the Mazandaran province, Iran between August 2010 and November 2011. The locations were chosen on the basis of random statistics for each zone of the province. Samples (approximately 500 g) were packed in sterile paper bags. The grain samples were stored in the laboratory at room temperature in the Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran, Iran. The mycological analysis started within a week after sampling. None of the samples contained visible signs of fungal contamination.

3.2. Isolation and Identification of Fusarium Species

According to the International Seed Testing Association (ISTA), samples were subjected to analysis by the hand halving method (17). Briefly, 100 paddy grains were surface sterilized by 1% sodium hypochlorite for 1 min and rinsed twice in sterile distilled water for 30s. The sterilized grains were cultured on potato dextrose agar (PDA) containing chloramphenicol (100 mg/L) at 27°C for 7 - 10 days. The developing fungal colonies were counted directly after incubation.

All *Fusarium* isolates were sub-cultured on PDA, SpezellerNährstoffarmer agar (SNA) and carnation leaf agar (CLA). PDA cultures were incubated at 27°C for 5-7 days, while CLA and SNA cultures were incubated at 25°C for 2-4 weeks. For morphological identification of the fungal isolates, we followed the criteria of Leslie and Summerell (18). Colony morphology was observed from cultures cultivated on PDA. To assess the morphology of macroconidia, microconidia, conidiogenous cells and the chlamydospores, we used the cultures incubated on SNA and CLA. In this study unless otherwise indicated, all the chemicals were purchased from Merck Co., Darmastdt, Germany.

3.3. Media and Single-Spore Isolation

Different *Fusarium* species cultures were transferred to 1 ml of sterile Tween 20 in water (1: 104, v/v) with a sterile wire loop. The suspensions were mixed by a vortex mixer, and streaked on agar/water plates with a sterile loop. Plates were incubated at room temperature overnight. For each culture, 3 germinated spores were picked and used to inoculate PDA slants and plates. Cultures were incubated at room temperature for 1-2 weeks on an alternating light-dark schedule. The slants and plates were washed with sterile distilled water to prepare the conidial suspensions. Each PDA medium (12 cm diameter) was inoculated with 1×10^7 of conidia and shaken once or twice daily for 3 days to distribute the inoculums. The cultures were incubated at 30°C for 10 days in the dark.

3.4. FB1 Analysis in Rice

The levels of FB1 in rice grains were determined by HPLC coupled to a fluorescence detector according to the procedure reported by Thakur and Smith (19) with some modifications. Briefly, 5 g of each sample was blended with 37.5 ml of acetonitrile: water (50: 50, v/v) in a blender at high speed for 5 min. The homogenate substrate was filtered through a Whatman No. 4 filter paper. Residues were re-extracted with 25 ml of acetonitrile: water (50: 50, v/v). Filtrates were combined, and a 5-ml aliquot was applied to a bond-Elut SAX cartridge (Varian Associates, Walnut, CA) that was conditioned with methanol (8 ml), followed by methanol: water (3: 1, 8 ml). Subsequently, the cartridge was washed successively with methanol: water (3: 1; 8 ml) and methanol (3 ml), and the toxin was eluted with 0.5% acetic acid in methanol (14 ml).

The eluate was evaporated to dryness under a stream of air at room temperature, the residue was suspended in 0.1 M sodium borate (200 ml), and aliquots (50 ml) of this solution were used for derivatization with 200 ml of o-phthaldialdehyde. Ten microliters of the derivatized solution was injected into the HPLC (Waters Associates, Milford, MA) exactly 1 min after derivatization. A C18 ODS reversed-phase column (150 × 4.6 mm, 5 μm particle size-Phenomenex, Torrance, USA) and an on-line corresponding guard column were employed under an oven tem-

perature of 35°C. Flow rate of the mobile phase was set at 1 ml/min.

Detection was based on elicitation of the FB1 derivative fluorescence with wavelength set at 229 nm and 442 nm for excitation and emission, respectively. The chromatogram retention time for FB1 derivative was approximately 9 min. Quantification was based on peak area measurement and comparison with FB1 standard. Method recoveries from the samples spiked with 0.1 to 3 mg of FB1/kg ranged from 78% to 94.5% for rice. The detection limit was approximately 0.04 mg/kg. Samples of rice that presented a peak within the FB1 retention area were confirmed by addition of the internal standard to cleaned samples at 1 and 3 mg FB1/kg.

3.5. Statistical Analyses

Chi-square (X2) and t-student tests were performed using the SPSS software (Version 15) to assay the mycoflora and the concentrations of FB1 in fresh and stored rice grains. A P value less than 0.05 was considered significant.

4. Results

Mycological analysis showed that fresh and stored rice grains were infected by 51 and 54 fungal colonies, respectively, representing no significant difference between the two kinds of rice samples ($P > 0.05$). The prevalence of fungal isolates indicated a slight increase over the 12 months of storage from about 48.6% infected samples at the beginning to 51.4% at 12 months of storage. *Aspergillus* was the predominant fungal genus in fresh (37.3%) and stored (40.7%) rice grains (Table 1).

The genus *Fusarium* was also detected in many samples (more than 38.3%), but with lower incidence; about 21.6% in fresh and 16.2% in stored samples. Species of Mucor and Rhizopus were encountered but in less than 19.6% and 9.8% of the fresh samples, and 16.7% and 11.1% of the stored samples, respectively. As illustrated in Figure 1, the most common *Fusarium* species in the rice samples was Fusarium spp with an occurrence of 35%, followed by *F. verticillioides* (25%), *F. solani* (15%), *F. oxysporum* (10%), *F. poae, F. expansum* and *F. equiseti* (5%). Among various *Fusarium* species, *F. verticillioides* was the predominant species in both fresh and stored samples. *Fusarium* occurrence showed no significant difference between thetwo samples as well as from one zone to another ($P > 0.05$). However, *Fusarium* incidence decreased throughout the storage period from 55% at harvest to 45% at 12 months of storage.

In the present study we used a practical method for the determination of FB1 in rice by HPLC coupled to a fluorescence detector. A widespread occurrence of FB1 in rice samples was observed for both fresh and stored rice (Table 2). Most samples (96.7%) collected were found to be fumonisin-positive with the mean levels ranging from not detected to 56.2 mg/kg for fresh samples and from 4.3 to 42.8 mg/kg for stored samples.

Table 1. Comparative Values of Various Fungi Isolated From Fresh and Stored Rice Grains Collected From Mazandaran Province, Iran in 2010-2011

Fungus	Rice Samples	
	Fresh, No. (%)	Stored, No. (%)
Alternaria alternata	2 (3.9)	1 (1.6)
A. flavus	2 (3.9)	5 (9.3)
A. fumigatus	6 (11.8)	5 (9.3)
A. niger	4 (7.8)	4 (7.4)
A. terreus	5 (9.8)	6 (11.1)
A. parasiticus	2 (3.9)	2 (3.7)
Scopolariopsis brevicaulis	0 (0)	1 (1.6)
F.verticillioides	3 (5.9)	2 (3.7)
F. equiseti	1 (2)	0 (0)
F. solani	2 (3.9)	1 (1.6)
F. oxysporum	2 (3.9)	0 (0)
F. expansum	0 (0)	1 (1.6)
F. poae	1 (2)	0 (0)
***Fusarium* spp**	2 (3.9)	5 (9.3)
***Penicillium* spp**	2 (3.9)	5 (3.7)
***Rhizopus* spp**	5 (9.8)	6 (11.1)
***Mucor* spp**	10 (19.6)	9 (16.7)
***Absidia* spp**	1 (2)	0 (0)
***Cladosporium* spp**	0 (0)	3 (5.6)
Saccharomyces cerevisiae	0 (0)	1 (1.6)
Rhodotorula rubra	1 (2)	0 (0)
Total	51 (100)	54 (100)

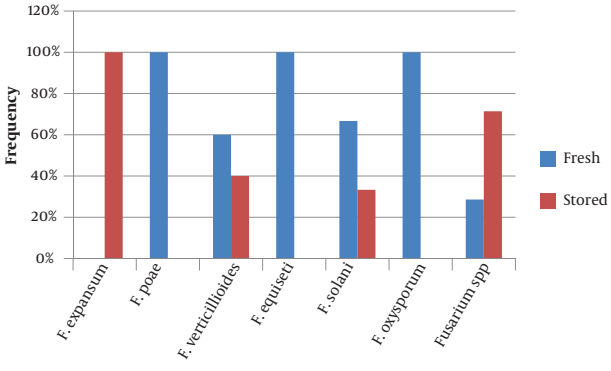

Figure 1. Comparative Values of Fusarium Occurrence Between Fresh and Stored Rice Samples in Different Zones of Mazandaran Province, Iran in 2010-2011

FB1 levels were higher in fresh rice (mean: 23.8 mg/kg) than stored rice (mean: 15 mg/kg) and changed through-

out the 12-month storage period, showing a decreasing trend in most zones. Although this decrease was not significant between the two kinds of samples tested, significant differences were observed in zones13, 14 and 15.

Table 2. Mean Total Fumonisin B 1 (FB 1) Levels in Fresh Rice Grains and Rice Samples Collected After a 12-Month Storage Period in 15 Different Zones of Mazandaran Province, Iran During 2010-2011

Zone	Rice Samples (Fumonisin B1, mg/kg)		
	No.	Fresh, Mean ± SD[a]	Stored, Mean ± SD
1	5	12.1 ± 0.9	4.3 ± 0.1
2	5	12.3 ± 1.0	6.3 ± 0.3
3	5	12.3 ± 1.0	7.1 ± 0.5
4	5	19.0 ± 1.7	8.7 ± 0.6
5	5	19.5 ± 1.8	9.5 ± 0.8
6	5	25.4 ± 2.1	11.5 ± 0.9
7	5	27.4 ± 2.2	25.4 ± 2.1
8	5	29.3 ± 2.0	15.9 ± 1.4
9	5	39.8 ± 2.9	19.1 ± 1.8
10	5	43.1 ± 3.3	23.1 ± 2.1
11	5	45.4 ± 3.5	27.5 ± 2.2
12	5	56.2 ± 3.8	28.1 ± 2.5
13	5	6.2 ± 0.3	56.2 ± 3.8
14	5	9.7 ± 0.7	33.3 ± 2.8
15	5	ND[b]	42.8 ± 3.2

5. Discussion

Rice is the most important staple food crop in Iran, which favors fungal contamination and mycotoxin production (20). During storage, the development of fungi, especially *Aspergillus*, *Fusarium* and *Penicillium* species, is an unresolved problem. They are responsible for quantitative and qualitative losses and under certain conditions these species can develop toxic metabolites such as fumonisins (21). The prevalence of fungal isolates indicated a slight increase over the 12 months of storage from 48.6% in fresh-harvested samples to 51.4% at 12 months of storage. During storage, the grains are susceptible to insects and their infestations disseminate the fungi and creates entry points in the grain for fungal infection (22).

In the present study *Aspergillus* and *Fusarium* genera were the most frequent fungal contaminants in fresh (37.3%, 21.6%) and stored (40.7%, 16.2%) rice grains, respectively. In addition, *Mucor* and *Rhizopus* species were identified in about 19.6% and 9.8% of fresh, and 16.7% and 11.1% of stored samples, respectively. Among different *Fusarium* species, *F. verticillioides* was the most frequent contaminant of the rice samples with an occurrence of 25%. However, *Fusarium* incidence decreased throughout the storage period, from 55% at harvest to 45% at 12 months of storage.

Previous studies showed that the major mycotoxigenic fungi in rice are *Aspergillus* spp (23), *Fusarium* spp (14) and *Penicillium* spp (21). In a study by Butt *et al*. (24), 27% of mycoflora were associated with rice grains and four fungal species namely *F. moniliforme*, *Alternaria* spp, *Helminthosporium* spp and *Curvularia* spp were isolated from different rice varieties. In several previous studies, fungi including *A. alternata*, *A. padwickii*, *A. longissima, A. niger, Curvulariaoryzae, C. lunata, Drechslera oryzae*, *F. miniliforme*, *F. semitectum*, *F. oxysporum*, *F. soalni, Pyriculariaoryzae, Phoma, Cercospora, Chaetomium, Sclerotium, Myrothecium* and *Colletotrichum* have been isolated from different rice samples collected from different countries (25-28).

The discrepancies in different studies were associated with various rice varieties, climate conditions, handling and mechanical damages by insects and rodents. Fumonisins belong to a large group of mycotoxins produced by fungi of the genera *Fusarium*. FBl is always the most abundant and toxic metabolite of this group of mycotoxins, representing about 70% of the total concentration in naturally contaminated foods, followed by FB2 and FB3 (30). We used a practical method for the determination of FB1 in rice by HPLC analysis using a fluorescence detector. Most of the rice samples (96.7%) were found to be positive for FB1 with mean levels ranging from not detected to 56.2 mg/kg for fresh and from 4.3 to 42.8 mg/kg for stored samples. The natural occurrence of fumonisins in rice was first reported by Abbas et al. (14) in Arkansas and Texas, USA, and there are some other reports on fumonisins in rice (29, 30).

In this report, it was shown by liquid chromatography using fluorescence detection (HPLC-FL) and enzyme-linked immunosorbent assay (ELISA) that 40% of infected rice with *Fusarium* species was positive for FB1 at level of 4.3 mg/kg (14). In a study conducted in Japan, Kushiro et al. (31) tested a sensitive detection method by liquid chromatography with tandem mass spectrometry (LC-MS /MS) for the determination of fumonisins in rice. This study could detect FB1 in rice grains at levels ranging from 0.061 to 0.101 mg/kg. Mallmann et al. (32) indicated positive results in 80% of rice samples assayed in Brazil. FB1 concentrations varied from 1.1 to 14.2 mg/kg (mean value: 5 mg/kg). The percentage of FB1 positive rice samples and the levels of FB1 obtained in our study are higher than those found in other countries.

This discrepancy could be due to differences in harvesting techniques, collection phase, storage conditions, test methods, source of *F. liseola* section isolates in soil and environmental conditions (temperature, relative humidity and rainfall). Mazandaran is one of the provinces in northern Iran, which has a favorable climate for fungal growth and mycotoxin production on food (with average annual temperature of 17.7°C and average humidity of 75.5%). The effect of rainfall and relative humidity on fumonisin levels showed a drastic variation during the harvesting season, which could have produced physiological stress on the crops (33). In addition, the results could be

related to the differences in agricultural practices in each zone, where farmers use a different handling process before and during harvest.

In the present study FB1 levels were higher in fresh rice (mean value: 23.8 mg/kg) than stored rice (mean value: 15 mg/kg) and changed throughout the 12-month storage period, showing a decreasing trend in most zones. Orsi et al. (34) observed an overall decrease of fumonisin content in stored maize after 140 days of a one year-storage period in Brazil. Our results are also in agreement with those of other investigators (35, 36) reporting that fumonisins were unstable in naturally contaminated maize and rice grains over long periods. There were indications that the fumonisins were unstable in naturally contaminated rough rice over long periods (35). Further studies are necessary to thoroughly explain this situation. Some factors including environmental conditions, intrinsic characteristics of stored products, chemical reactions and microbial competition are suspected (37).

Many species of bacteria, fungi and yeasts have been shown to enzymatically degrade mycotoxins during storage (35, 38, 39). Kim et al. (40) also suggested that fumonisin molecules might bind with the starch of the product during storage to form a complex, which is not detectable. In addition, they suspected the moisture content of the product, its texture and metal ions influence fumonisin loss. Scott et al. (36) reported that reaction of FB1 with reducing sugars such as D-glucose is likely to explain the rapid fumonisin loss observed in maize starch. In the present study, a positive relationship (not significant) was observed between the FB1 levels in rice and *Fusarium* occurrence among fresh and stored samples. In fact, there is total dependence of the isolate with regard to the accumulation of fumonisins in rice experimentally contaminated with different isolates of fumonisin producing species (40).

In conclusion, this study demonstrated that rice grains could potentially contain various toxigenic fungi, in particular *Aspergillus* and *Fusarium* species, and high levels of FB1 toxin. Further study on the decomposition of FB1, as the most abundant fumonisin, during processing as well as cooking is also required to assess and minimize the exposure to FB1 in populations who live on rice as a staple diet.

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Authors' Contribution

None Declared.

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