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RESEARCH ARTICLE

Molecular identification of aflatoxigenic *Aspergillus* species in dried nuts and grains collected from Tehran, Iran

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Abstract

Introduction Agricultural commodities contaminated by molds and mycotoxins can be considered as public health problems in less developed countries, particularly in Iran. Hence the main purpose of this study was to identify mold fungi and molecular analysis of the most important species of aflatoxin-B1-producing *Aspergillus* species in some dried nuts and grains in local markets in Tehran.

Materials and methods Two hundred fifty samples of wheat, rice, corn, pistachios, and peanuts were collected from the five different locations of Tehran between January 2018 and January 2019. The samples were analyzed by using direct seed inoculation method and grain crushing method. Fungal strains were identified as *Aspergillus* spp. on the basis of morphological characters and further confirmed by using of β-tubulin gene sequencing. To differentiate between aflatoxigenic and non-aflatoxigenic *Aspergillus* spp., the isolates were screened for the presence of aflatoxigenic genes (*nor-1*, *ver-1*, *omtA*, and *aflR*).

Results One-handed forty-eight aflatoxigenic *Aspergillus* isolates (144 *A. flavus* and 4 *A. parasiticus*) were identified and *aflR* gene was the most frequent gene in these species. Five isolates (4 *A. flavus*, 1 *A. parasiticus*) had quadruplet pattern, 64 isolates (63 *A. flavus*, 1 *A. parasiticus*) had more than 1 gene and 39 isolates (38 *A. flavus*,1 *A. parasiticus*) did not have any genes.

Conclusion According to the contamination of dried nuts and grains by some aflatoxigenic fungi, an extensive surveillance is necessary to provide a wider view on these products. Moreover, effective and efficient aflatoxin control program requires identifying and managing key elements that are effective in reducing mycotoxin production at farm level or in storage conditions.

Keywords Grains · Nuts · *Aspergillus* · Aflatoxin

Introduction

In all over the world, especially in Iran, food gradients including wheat, corns, pistachio nuts, and peanuts are considered as the main nutrient sources [1]. These products have the high susceptibility to mycotoxin contamination which is a serious threat for animal and human health [2]. Mycotoxins can be produced by several species of molds, such as *Fusarium*, *Penicillium* and *Aspergillus* species [3]. Improper pre-harvest or post-harvest conditions of agricultural products are the key factors in the contamination with these fungi [4]. The percentage of cereals contaminated by mycotoxins has been estimated approximately 25 % around the world [5]. Aflatoxins are produced widely by *Aspergillus flavus* and *Aspergillus parasiticus* belonged to *Aspergillus* section *Flavi* [6]. There are reports from different parts of the world confirming that aflatoxicosis, as a food-borne disease, causes poisoning, sickness, and death in humans and animals [7].

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Among different forms of aflatoxin (B1, B2, G1 and G2), International Agency for Research on Cancer (IARC) has considered aflatoxin B1 as the first group of human carcinogen [8]. Several structural genes in the aflatoxin gene cluster comprising *nor-1*, *ver-1*, and *omtA* encode enzymes of norsolorinic acid reductase converting norsolorinic acid to averantin, versicolorin A dehydrogenase converting versicolorin A to sterigmatocystin and sterigmatocystin-o methyltransferase converting dimethyl sterigmatocystin to sterigmatocystin, respectively. Furthermore, the *aflR* gene encodes a regulatory factor activating transcript of some structural genes in aflatoxin gene cluster [9]. Screening and identification of fungal isolates on the basis of macroscopic and microscopic characteristics are difficult, especially in the case of morphologically-similar species [10]. In recent years, although advantage of the phenotypic identification method is undeniable, molecular methods due to their rapidity, sensitivity and specificity are employed for identification of fungi. The present study was conducted to investigate the distribution pattern of aflatoxigenic *Aspergillus* species and their status regarding the presence of aflatoxigenic genes, in wheat, rice, corn, pistachio, and peanut in local markets of Tehran, Iran.

Material and method

Collection of samples

In this descriptive cross-sectional study, 250 samples comprising of wheat (n = 50), rice (n = 50), corn (n = 50), pistachios (n = 50), and peanuts (n = 50) were collected from the five different locations of Tehran (North, South, East, West, Center) between January 2018 and January 2019.

Cultivation, isolation, and identification

Collected samples were cultured by using two methods namely direct implantation and seed grinding on Sabouraud dextrose agar (SDA) plates. In the direct method, 3–5 out of each of samples were inoculated on the SDA containing antibiotic chloramphenicol. In the method of seed grinding, 1 g of grounded samples of wheat, rice, corn, pistachio, and peanut separately was mixed with 10 mL of sterile distilled water then 100 μl from prepared suspension was transferred to SDA containing chloramphenicol. Culture plates of two methods were incubated at 26 °C for 7 days. During this time, the growth of *Aspergillus* spp. was controlled daily. All strains were preliminarily identified to the species level based on morphological characteristics; subsequently, their identity was confirmed by molecular approaches.

DNA extraction

The isolation of chromosomal DNA from filamentous fungi was done according to the used technique of Yelton et al. with some modification [11]. The quality and quantity of DNA was analyzed by resolving the samples on gel electrophoresis [12].

Molecular identification

All *A. flavus* and *A. parasiticus* isolates were identified by microscopic and macroscopic characteristics; subsequently, their identity was confirmed by DNA sequencing of the partial β-tubulin (BTU) gene, using the primers Bt2a (5'-GGT AAC CAA ATC GGT GCT TTC-3') and Bt2b (5'- ACC CTC AGT GTA GTG ACC CTT GGC-3') [13]. A typical PCR mixture contained: 2 μL DNA template, 5 μL Taq polymerase buffer, 1.25 μL primer (120 pmol mL^{-1} each), 25 μL H2O and 0.1 μL Taq polymerase (5 U μL^{-1}). Briefly, amplification of the BTU gene was performed with 2 min for initial denaturation in the start of one cycle at 94 °C, followed by 35 cycles at 94 °C for 60 s, 54 °C for 60 s, and 68 °C for 60 s, with a final 10-min extension at 68 °C. Sequence data obtained were compared with those deposited in the NBLAST algorithm (http://blast.ncbi.nlm.nih.gov) and the local database at the CBS-KNAW Fungal Biodiversity Centre (Utrecht, The Netherlands).

Multiplex PCR reaction

The sequences of the primers of *aflR*, *nor-1*, *ver-1*, and *omtA* genes are shown in Table 1 [14]. Each PCR reaction mixture with a total volume of 20 μL was contained 10 ng genomic DNA extracted from fungal isolates, 0.4 μL sense and 0.4 μL antisense primers of *aflR, nor-1*, *ver-1*, and *omtA* genes, 2x (dye mix) ready to use PCR master mix containing Taq DNA polymerase (Merck). The amplification of desired genomic regions was done by thermal cycler (Techne) according to the temperature-time program optimized including 6 min for initial denaturation in the start of one cycle at 95 °C, followed by 6 cycles of 45 s at 95˚C, 45 s at 58.2˚C and 80 s at 72˚C, then 30 cycles of 60 s at 95˚C, 60 s at 56˚C, 60 s at 72˚C. While final extension of 10 min, were given at 72˚C. Amplified DNA fragments were electrophoresed on 1.2 % agarose gel stained with ethidium bromide [15].

Results

In general, 473 fungal isolates were recovered from the 250 collected samples (Table 2). From this fungi, 285 isolates were identified as *Aspergillus* spp., 148 of them were confirmed to be aflatoxigenic speices, i.e. *A. flavus* (144, 50.5 %), and *A. parasiticus* (4, 1.4 %) by molecular method.

Table 1 Target genes, primer sequences and PCR product size of Aflatoxin-producing genes

Primer code	Target gene	Primer sequences	PCR product size (bp)
Nor	Nor.1for	ACCGCTACGCCGGCACTCTCGGCAC	399
	Nor.2rev	GTTGGCCGCCAGCTTCGACACTCCG	
Ver	Ver.1for	GCCGCAGGCCGCGGAGAAAGTGGT	537
	Ver.2rev	GGGGATATACTCCCGCGACACAGCC	
Omt	omt.1for	GTGGACGGACCTAGTCCGACATCAC	797
	omt.2rev	GTCGGCGCCACGCACTGGGTTGGGG	
AflR	aflR.1for	TATCTCCCCCCGGGCATCTCCCGG	1032
	aflR.2rev	CCGTCAGACAGCCACTGGACACGG	

These strains isolated from 39 (26.35 %) corn, 36 (24.32 %) rice pieces, 29 (19.60 %) pistachio, 26 (17.57 %) peanut and 18 (12.16 %) wheat samples. Moreover, all *A. parasiticus* isolates were identified in wheat samples in center of Tehran. The DNA fragments amplified by using multiplex PCR corresponding to *aflR*, *nor-1*, *ver-1*, and *omtA* genes have given four bands of 400, 537, 797 and 1032 bp on gel electrophoresis (Fig. 1). Regarding the frequency of aflatoxin biosynthesis genes, *aflR* gene was detected in 86 (58 %) *A. flavus* and 3 (2 %) *A. parasiticus* isolates, *nor-1* gene was detected in 43 (29 %) *A. flavus* and 1 (0.7 %) *A. parasiticus* isolates, *ver-1* gene was detected in 32 (22 %) *A. flavus* and 1 (0.7 %) *A. parasiticus* isolates and *omtA* gene was detected in 30 (20 %) *A. flavus* and 1 (0.7 %) *A. parasiticus* isolates (Fig. 2). The investigation of gene patterns indicated that the presence of four genes of aflatoxin biosynthetic pathway was observed in five isolates (1 *A. parasiticus* and 4 *A. flavus*), 64 isolates (63 *A. flavus*, 1 *A. parasiticus*) had more than 1 gene and 39 isolates (38 *A. flavus*,1 *A. parasiticus*) did not have these four genes.

Table 2 The frequency of fungi isolated from rice, corn, wheat, pistachio, and peanut samples using two methods of direct seed insemination and grain crushing methods

Isolated fungi	Number (%) of isolated fungi	
	grain crushing	direct seed insemination
Aspergillus spp.	68 (61.2%)	217(59.9%)
Mucor spp.	22 (19.8%)	85(23.4%)
Rhizopus spp.	6 (5.4%)	56(15.4%)
Penicillium spp.	8 (7.2%)	3(0.8%)
Monilia spp.	-	1(0.2%)
Fusarium spp.	4(3.6%)	-
Paecilomyces spp.	3(2.7%)	-
Total	111(100%)	362(100%)

Discussion

Aflatoxins, known as secondary metabolites, are produced by some *Aspergillus* spp. These compounds are the public-health challenge due to their importance in mutagenicity, carcinogenicity on human and animals. The analysis of some agricultural foodstuffs in different production processes has indicated the presence of aflatoxins in these products leading to the consignment rejection of these products via consumers [16, 17]. In this investigation, we demonstrated that distribution pattern of mold fungi associated with them from five different areas of Tehran city. Our findings showed the highest and lowest prevalence was related to corn and wheat samples. By contrast, the report of Lutfullah et al. observation on some cereals and beans of Pakistan indicated that wheat samples had the highest prevalence of contamination. The cause of this difference may be inappropriate conditions of pre-harvest and post-harvest of crops [18]. In studies conducted in Sanandaj and Sari (Iran) on peanuts and pistachio nuts according to morphological profiles indicated that the *A. flavus* species was more common than other *Aspergillus* spp., confirming our results [19, 20]. While, in other studies in Isfahan, Rafsanjan and Kerman cites of Iran on pistachio samples, the most predominant *Aspergillus* spp. isolated were *A. niger* and *A. flavus*, respectively [21]. Also in the study of Furlung et al. the genus *Aspergillus* was not present among the fungi isolated from wheat in Brazil [22].

In order to reduce the risk of aflatoxin consumption, the isolation of agricultural commodities contaminated by aflatoxin-producing fungi is necessary. From this regard, because of

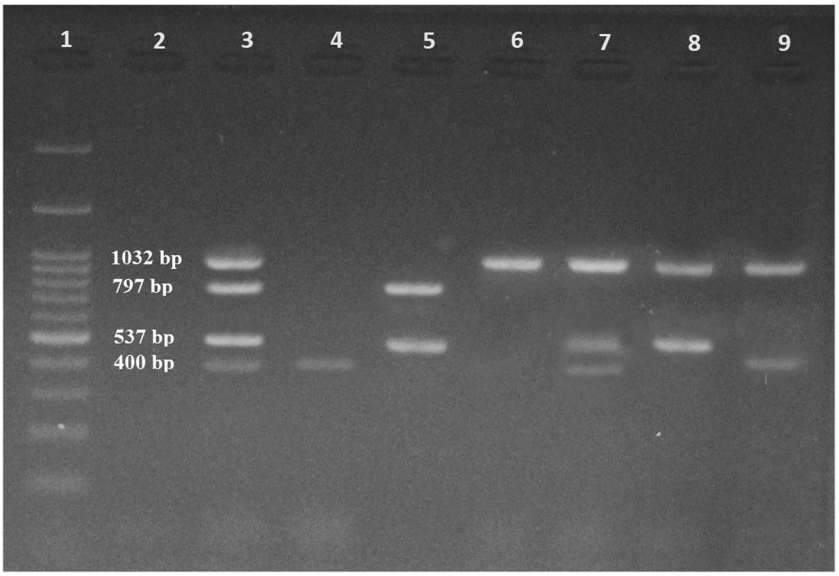

Fig. 1 The DNA fragments amplified by using multiplex PCR corresponding to *nor.1* (400 bp), *ver.1* (537 bp), *omtA* (797 bp) *and aflR* (1032 bp) genes have given four bands on gel electrophoresis. 1: DNA Ladder, 2: Negative Control, 3: Positive Control, 4–9: Samples

remarkable merits of PCR-based methods such as simplicity, accuracy, sensitivity and rapidity, they are useful for the detection of four effective genes in aflatoxin biosynthesis pathway [23]. In the study of Criseo et al. in contrast to our study, the *nor-1* gene was the most representative and the *aflR* gene had the lowest incidence among the four aflatoxin genes tested [24]. Studies conducted to evaluate the prevalence of four aflatoxin genes (*aflR, nor-1, ver-1*, and *omtA)* with the use of multiplex PCR method indicated that in non-aflatoxigenic *A. flavus* strains isolated from food and feed samples in Italy and *A. flavus* and *A. parasiticus* strains isolated from maize samples in Kenya, the *nor-1* gene was known as the most prevalent gene [25]. Another study showed that *omtA* gene was the most representative gene in *A. flavus* and *A, parasiticus* isolates in Egyption chili [9, 15, 23]. According to previous investigations, although all aflatoxigenic strains had complete pattern of four genes, some strains with compete pattern of four tested genes due to molecular defects at levels of transcription and protein translation were non-aflatoxigenic strains [24]. From this regard, it is unable to take the presence of complete pattern into account a difference between aflatoxigenic and non-aflatoxigenic strains [15, 24].

In conclusion, according to the contamination of dried nuts and grains by some aflatoxigenic fungi, an extensive surveillance is necessary to control of these products. Multiplex PCR method can be used for screening and primary isolation when faced with large numbers of samples.

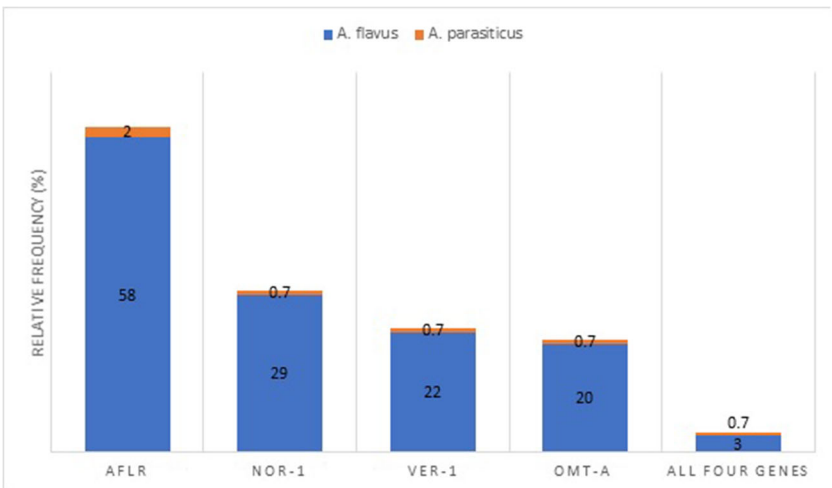

Fig. 2 Frequency of *nor-1, ver-1, omtA* and *aflR genes* involved in aflatoxin biosynthesis using the multiplex PCR assay

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Declarations

Conflict of interest There is no conflict of interest between authors in this study.

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