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Two Important *Fusarium* in Esophageal Cancer, *Fusarium verticillioides* and *Fusarium proliferatum*: Mycoflora Isolation and Molecular Characterization in Iranian Feed and Corn

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ABSTRACT

Mycoflora survey in feed permits estimation of the feed deterioration and the risk of mycotoxin contamination. Fusarium species are one of the noticeable fungal in Iran and their toxigenic ability is a health hazard for both animals and humans. In this work, we took samples from five provinces of Iran. We isolated the endogenous fungi using the Freeze blotter technique and separated exogenous fungi implementing the method of flotation with dichloran-chloramphenicol agar (DCPA) and Malachite green agar (MGA). Also, we used PCR assays and the sequencing of TEF1-α gene (Translation Elongation Factor 1-α) to recognize the Fusarium species in isolates from various areas of Iran. Mycological analyses showed the percentage of fungal species including Aspergillus spp. (26.5%), Fusarium spp. (19.6%), Penicillium spp. (17%), Mucor spp. (3.2%), Rhizopus spp. (1.4%), Alternaria spp. (1.8%), and Cladosporium spp. (4%). Fusarium verticillioides were the most common species (70.3% of Fusarium isolates and 13.8% of the whole isolations), and the highest incidence was in Qazvin with 22.5%.

Key words: *Fusarium*, Exogenous fungi, Freeze blotter, PCR, Sequencing

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INTRODUCTION

Fusarium species and their mycotoxins are one of the most referable and noticeable fungi in Iranian feeds and feed ingredients that have gained increasing attention in previous studies. Notably many experts such as Chehri et al., Ghiasian et al., Hadiani et al., Shephard et al., etc., performed remarkable studies on *Fusarium* in their researches [1-4].

Fumonisins, zearalenone (ZEN), trichothecene group (including diacetoxyscirpenol (DAS), deoxynivalenol (DON), and T-2 toxin), moniliformin, and fusaric acid are some important *Fusarium* mycotoxins [5]. WHO-IARC (WHO-International Agency for Research on Cancer) estimated the carcinogenic ability of mycotoxins, and fumonisins and ranked them as potential carcinogens (Group 2B). Trichothecenes and zearalenone, though, were not ranked as human carcinogens (Group 3) [6]. But, ZEN was observed in the blood of children with precocious sexual development and airborne toxicosis with trichothecene can cause "sick building syndrome" [7]. Also, exposure to FB1 during the first trimester and before the pregnancy emerged to increase the hazard of neural tube defects (NTD) [8,9].

In the report of Manual of Sanitary Seed Analysis (2009), techniques generally advised to identify pathogens on seeds were agar culture medium and Blotter test [10]. The suitable method for evaluating the surface contaminated by fungal mycoflora (exogenous mycoflora) is flotation method (then culture in Malachite green agar and dichloran-chloramphenicol agar). The Freeze Blotter (FB) method is a fine method for isolation of internal fungal mycoflora (endogenous mycoflora) [11].

Some benefits of Freeze Blotter (FB) method are the identification of different fungi conducted by seeds at the same time, comfortable in setting up and low cost [10].

Although the global sharing of toxigenic *Fusarium* species is extensive, finding an universality of any species-specific PCR assay is always a challenge specifically in *Fusarium* species from different crops and/or geographic areas. The genes of *TEF1-α* (translation elongation factor 1-α) show a high level of sequence polymorphism between closely associated species compared with other genes like histone H3, b-tubulin and calmodulin. For these reasons, the selective marker as a single-locus recognition instrument in *Fusarium* species is *TEF1-α* [12].

Perhaps, *F. verticillioides* and *F. proliferatum* are the most prominent producers of fumonisin B1 (FB1) as they are the usual pollutants of corn in various location of the world [13].

F. verticillioides (syn *Gibberella moniliformis*, *teleomorph G. fujikuroi*) happens across the globe and in both tropical and temperate areas of feed integrant. *F. verticillioides* might be the most usual pathogen on maize cobs, maize kernels and stalks all over the world [14].

F. proliferatum is a fungus with a global sharing that has been linked to various diseases in urgent economical plants such as bananas and corn. It can create a spread infection in immunocompromised patients and abscesses in the body when trauma is because of a plant. Although *F. proliferatum* in contact lens maintenance solution was remarked as an agent that can cause *Fusarium keratitis* [15].

MATERIALS AND METHODS

Sampling method

The sampling was based on the ISTA (International Seed Test Association) protocol [16]. All 69 samples were captured randomly from different vertical and horizontal places during 2016 to 2017. Samples were transferred in paper bags and directly transferred to the laboratory, then kept at 5°C ± 1°C.

Isolation of fungus

In Blotter test with freezing, for antisepsis, samples were washed by sodium hypochlorite solution (1%) for 3 min. Samples were moved on incubation chamber of acrylic plates and stored in 20°C with shifting 12 h period of fluorescent white light and darkness. Then, samples for freezing and temperature shock sent to -20°C for 24 h. Thereafter, they were incubated for 5 days at 20°C and 25°C [10].

In the flotation method, 160 grams of samples (four series, each contains 40 gram) were examined. A suspension consists of 40 grams of samples; 8 ml sterile distilled water, Tween 80 (0.005%/ml), streptomycin (10 mg/ml), penicillin (20000 U/ml), was shaken for 1 minute then unshaken for 15-30 minutes. 0.5 ml of supernatant solution was cultured on two plates of Malachite green agar and on two dichloran-chloramphenicol agar (DCPA) by streak method. The plates for 4-10 days were incubated in the dark at 28°C. Dichloran-chloramphenicol agar (DCPA) culture contains 15 g of bacteriological peptone, 1 g of K_2HPO_4, 0.5 g of $MgSO_4.7H_2O$, 20 g of agar and 1 liter of distilled water. To this basal medium, chloramphenicol (200 µg/ml) or dichloran (2 µg/ml) was supplemented to made chloramphenicol-peptone agar and dichloran-peptone agar, respectively. Adjustment to the composition of DCPA was adapted with the different measure from 0 to 25 µg of dichloran per ml, 0 to 1.0 g of $MgSO_4.7H_2O$ per liter, and 5 to 30 g of bacteriological peptone per liter. All media were sterilized at 121°C for 15 min; final pH index was in the range of 6.0 to 6.4 [17].

For Malachite green agar (MGA) culture, samples were washed in a sodium hypochlorite solution (1%) for 3 min. Malachite green agar contains 15 g of bacteriological peptone, 1 g of K_2HPO_4, 0.5 g of $MgSO_4.7H_2O$, 20 g of agar, 1 L of distilled water and malachite green 2.5 ppm in 1 L of distilled water. All media were sterilized at 121°C for 15 min [18].

The product fungal colonies from each above technique were kept and later subcultured in PDA (Potato Dextrose Agar).

Morphological detection

We evaluated macroscopic features (like powdery appearance on PDA) and all microscopic feature of single-spore isolate (capture from PDA culture) such as chlamydospore exist, polyphialide and monophialides exist, form of conidia of aerial mycelium or microconidia globose/napiform, and sporodochial conidia or macroconidia detected under nUV [19,20].

DNA extraction

Based on study by Kachuei et al. [11], the principle protocol for extracting the DNA of various funguses includes the following steps. First, mycelial pieces were flash-frozen in liquid nitrogen and crushed to a fine powder in a porcelain mortar, then this powder was hung in DNA extraction buffer containing 50 µl of proteinase-K (20 mg/ml), 3% SDS, 50 mM EDTA, and 50 mM Tris-HCl (pH 8.0). Thereafter, (for eliminating of cellular debris) the suspension was incubated at 65°C for 1 h and centrifuged at 2500X g for 15 min. After attachment of 25 µl RNase H (10 mg/ml), the product was incubated at 37°C for 30 min, and the product extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and then with chloroform-isoamyl alcohol (24:1). In the next step equal volume of isopropanol was added to product and centrifuge at 15000X g for 30 min. At last, The DNA was purge with 70% ethanol and resuspended in distilled water.

PCR and DNA sequencing

DNA of the mentioned *Fusarium* isolate was used in PCR with the *Fusarium* general primers [21].

Amplification was accomplished 25 µl in a final volume. The reaction involved 12.5 µl of Master Mix (Ampliqon; Taq DNA Polymerase Mix Red-$MgCl_2$ 1.5 mM/2 mM), 1 µl (50-100 ng) of template DNA, 1 µl of each primer, and 9.5 µl distilled water. The PCR condition for EF1 and EF2 including the primary denaturation at 94°C for 5 min, 34 cycles each of 30 s at 94°C, 40 s at 53°C, 45 s at 72°C, and a final extension at 72°C for 5 min. The PCR product with an approximate size of 568-bp was sequenced (Table 1).

Amplified PCR products were detached on an agarose gel (1% w/v) in 0.5X TBE buffer at 81 V for 40 min.

Table 1: Sequences of primers employed in the research

Primer Name	Sequences of primer	Annealing temperature
ITS1	TCCGTAGGTGAACCTGCGG	56°C
ITS4	TCCTCCGCTTATTGATATGC	
EF1	ATGGGTAAGGAGGACAAGAC	52°C
EF2	GGAAGTACCAGTGATCATGTT	

The 54 isolates were recognized to belong to *Fusarium* species, sequenced by means of TEF-1α gene sequences. The gene was amplified using EF1 (5'-TGGGTAAGGAGGACAAGAC-3') and EF2 (5'-GGAAGTACCAGTGATCATGTT-3') primer pair [21]. After sequencing, all sequences were compared with the existing sequences present in the NCBI database (www.ncbi.nlm.nih.gov/) using BLAST.

Statistical analysis

Statistical analysis was calculated using SPSS software (version 16). P-value calculated by T-test and T2 method. A p-value less than 0.05 were considered significant.

The isolation frequency (Fq), relative density (Rd) [22] and the incidence of genera and species isolated were calculated as follows (Equations 1-3):

Frequency (%)=(Number of samples in which a species/ genus occurred)/ Total number of samples × 100 (1)

Relative density (%)=(Number of isolates of a species/ genus)/(Total number of fungi/genus isolates) × 100 (2)

Incidence (%)=(Number of isolates of a genus/species in the province)/(Total number of isolates in the province) × 100 (3)

RESULTS AND DISCUSSION

Morphological identification

From 69 samples under examination, 224 fungal isolates were collected by FM method and 51 fungal isolates were recovered by FB method. The isolates belonged to 14 species of fungal. The percentage of fungal species by FM-FB methods was; *Penicillium* spp. (16.5-19.6%), *Aspergillus* spp. (25.8-29.4%), *Fusarium* spp. (22.3-7.8%), Unknown isolates (16.9-17.6%) was major fungi using FM-FB methods. However, dematiaceous fungi such as *Alternaria* spp. (0.04-7.8%) and *Cladosporium* spp. (4.4-1.9%) were the minor fungi. In Table 2, fungal genera based on morphological identification have been listed. Most of the Unknown isolates based on morphological definition were *Fusarium*, but since in amplification with EF1 and EF2 weren't positive we decided to call them "Unknown spp." (Figure 1).

Our results were akin to those of [23], and the predominant isolate in our research was *Aspergillus* spp. However, *Fusarium* spp. was the predominant isolate in most of the researches [22,24,25].

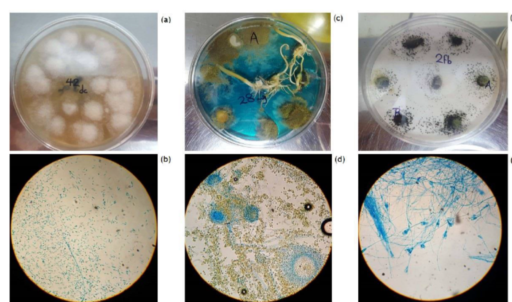

Figure 1: (a) Culture on DCPA after 9 days: *Fusarium* spp; (b) *Fusarium* spp. morphological features; (c) Culture on MGA after 7 days: *Aspergillus* spp; (d) *Aspergillus* spp. morphological features; (e) Culture by freeze blotter method, 5 days after temperature shock: *Penicillium spp*; (f) *Penicillium* spp. morphological features

Table 2: The fungal genera recovered from corn and animal feed collected of silos in five provinces in Iran, during 2016-2017

	Markazi province				Tehran province				Qazvin province				Qom province				Alborz province				Total		
	FMa		FBb		FM		FB		FM		FB		FM		FB		FM		FB		FM	FB	Both Method
Species	feed	corn	feed	corn	feed	corn	feed	corn	feed	corn	feed	corn	feed	corn	feed	corn	feed	corn	feed	corn			

Acremonium spp.	-	-	-	-	-	-	-	-	-	-	-	-	-	4	-	-	-	1	-	-	5	-	5
Alternaria spp.	-	-	3	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	1	-	1	4	5
Aspergillus spp.	5	6	2	-	7	6	1	2	-	4	1	-	1	3	1	-	13	13	7	1	58	15	73
Cladosporium spp.	-	-	-	-	2	-	-	-	1	-	-	-	1	1	1	-	5	-	-	-	10	1	11
Fusarium spp.	1	1	1		2	5	-	-	3	7	-	-	2	8	1	-	9	12	2	-	50	4	54
Lichtheimia spp.	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	1	3	-	2	3	5
Mucor spp.	-	2	-	-	1	-	-	-	-	2	1	-	-	-	-	-	2	1	-	-	8	1	9
Paecilomyces spp.	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	2
Penicillium spp.	1	-	1	-	10	1	-	-	-	2	1	-	2	-	1	-	16	4	4	4	37	10	47
Phialohora verrucosa	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	1	-	1
Rhinocladiella	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	1	-	1
Rhizopus spp.	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	3	-	-	4	-	4
Scopulariopsis spp.	-	-	-	-	-	-	-	-	-	-	-	-	2	-	1	-	5	-	2	-	7	3	10
Sporotrichum spp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	1	-	1
Unknownsp p.	1	1	4	-	2	5	-	-	4	3	-	-	5	-	3	-	14	3	2	-	38	9	47
Total Isolation	8	10	11	-	26	17	1	2	8	20	3	-	15	16	8	-	66	38	21	5	224	51	275
Total sample	5	-	-	-	15	-	-	-	7	-	-	-	10	-	-	-	32	-	-	-	69	-	-

FM: Flotation Methods; FB: Freeze Blotter method

The relative density for internal mycoflora and external mycoflora were 18.6% and 81.4%, respectively. External mycoflora was the predominant mycoflora in most of the fungal species. The incidence of internal contamination with *Alternaria* spp. and *Lichtheimia* spp. was only higher than external contamination.

Alternaria species create numeral mycotoxins containing tenuazonic acid, altertoxins I, II, III, alternariol, altenuene, alternariol monomethyl ether, and other less toxic metabolites. There are many reports on the mutagenicity and genotoxicity of alternariol, and alternariol monomethyl ether. Alternariol has been recognized as a topoisomerase I and II poison that probably tend to the destruction of DNA integrity in human colon carcinoma cells [26]. *Lichtheimia* (formerly *Absidia*) species is known as the second to the third most prevalent agent of mucormycoses in Europe. In addition, *Lichtheimia* species can result in occupational hypersensitivity pneumonitis termed Farmer's lung disease (FLD) [27].

The highest incidence of external mycoflora of feed and corn observed in Tehran's feed and corn of Qazvin with 56.5% and 64.5% respectively and the lowest external mycoflora of feed and corn observed in the feed of Qazvin and corn of Alborz with 25.8% and 29.2% respectively.

The highest incidence of internal mycoflora of feed and corn was observed in the feed of Markazi and corn of Tehran with 37.9% and 4.3% respectively. Also, internal contamination of corn was not detected in Markazi, Qazvin, and Qom provinces. The lowest internal mycoflora of feed was in Tehran with 2.1%.

Among the Zygomycetes, fungi of the genera *Rhizopus* and *Mucor* were found at incidence levels of 1.4% and 3.2%, respectively. Ghiasian et al. [22] reported that the level of *Rhizopus* and *Mucor* in Iranian maze was 4.6% and 1.3%. Also, Bujari et al. (1993) [28] isolated *Mucor cf. indicus* and *Rhizopus oryza* from all maize samples they tested form Mazandaran and Alborz in Iran. However, in our study *Rhizopus* species isolated only from 5.7% of samples and did not isolated from all samples.

Fungi of the order *Mucorales* consisted of 6 families. *Mucormycosis* represents a group of life-threatening infections caused by fungi of the order *Mucorales*. Between the families of the order *Mucorales Rhizopus* is the most prevalent, while Mucor is a scarce cause [29].

Penicillium species were isolated from feed and corn, and relative density of *Penicillium* spp. was 4% and 13% (in corn and feed). The highest and lowest incidence of *Penicillium* species occurred in Tehran and Markazi provinces (23.9%) and (6.8%), respectively. Hesseltine et al., [30] found that 28.6% of samples of maize grain from in North Carolina, USA, were contaminated with *Penicillium* species. Also, Ghiasian et al. [31] report that 23.7% of cow feeds in Hamadan, Iran, was contaminated with *Penicillium* species. While González et al., [25] found only 21.6% of maize grains to be contaminated by *Penicillium* species in Argentina and Ghiasian et al., [22] reported that only 4.5% of Iranian maize harvested in the major production locations contaminated by *Penicillium* species.

Mycotoxins created by the several species of *Penicillium* including citreoviridi, citrinin, griseofulvin, luteoskyrin, ochratoxin A, patulin, penicillic acid, PR-toxin, roquefortine, rugulosin, verrucosidin, verruculogen, viridicatumtoxin, and xanthomegnin. The most toxic and most important mycotoxin is ochratoxin A. It has been detected to be nephrotoxic, immunosuppressive, carcinogenic and teratogenic in all experimental animals. In IARC classification, ochratoxin A is known as a complex possibly carcinogenic to humans (Group 2B) [7]. To establish the potential toxigenic risk of these fungi on feed and maze, it will be necessary to identify the *Penicillium* cultures isolated to species level.

Within the genus *Aspergillus*, the potentially toxigenic *A. flavus* was the most widely recovered. This species was isolated from 16% of feed and corn. The other *Aspergillus* sp. identified was *A. niger*, which was isolated from 0.7% of corn and feed. The incidence of *A. flavus* in Markazi, Tehran, Qazvin, Qom, and Alborz was 34.3%, 17.3%, 12.9%, 5.1%, and 15.3% respectively. The incidence of *A. niger* in Markazi and Qom was 3.4% and 2.5% but in other provinces, *A. niger* did not contaminate corn and feed. Also, the incidence of *A. nidulans* in Markazi, Tehran, and Alborz was 3.4%, 2.1%, and 3.07% respectively, but in Qazvin and Qom *A. nidulans* did not contaminate corn and feed.

In North Carolina, USA, *A. flavus* and *A. niger* have been reported to infect 31.8% and 10.9% of grain, respectively [30]. Khosravi et al., [32] reported that *Aspergillus* contaminated 56% of feed in Qom and *A. flavus* was identified as predominant species with 46% frequency between the *Aspergillus* species. Also, *A. flavus* and *A. niger* contaminated 68% to 78% and 14% to 21% of Broiler Feeds in Kermanshah Province, Iran, respectively [33]. Based on FM-FB techniques, between the isolated *Aspergillus* species, the *A. niger* (2.3-6.3%), *A. fumigatus* (4.4-4.1%), and *A. flavus* (5.9-6%) were reported by Kachuei et al. [11].

Aflatoxin B1 and Aflatoxin B2 are highly toxic, teratogenic, carcinogenic, and mutagenic metabolites created usually by *A. flavus* and have been incriminated as causative agents in human hepatic and extrahepatic carcinomas. Aflatoxin B1 is 'Carcinogenic to humans' and has been estimated as a group 1 carcinogen. *A. niger* that can produce ochratoxin A [22]. According to Marin et al., [34] when *A. flavus*, *A. niger* and *A. ochraceus* (Bainier) Thom are members of competing for microflora, they may obstruct the synthesis of fumonisins by *Fusarium* isolates, or they probably decrease the mycotoxin as soon as it is created. It has been shown that the production of fumonisins by *F. verticillioides* and *F. proliferatum* could be changed by other maize fungi.

PCR assays and sequencing of *TEF1-α* gene

The outcome of PCR assays based on ITS, EF sequences recommended that 54 isolates belonged to the *Fusarium* species. A BLAST explore for resemblance displayed that partial *TEF1-α* gene sequences of isolates had a high similarity with *Fusarium verticillioides* and *Fusarium proliferatum* (Table 3). *TEF1-α* gene sequence of isolates morphologically determined as *Fusarium* species.

Table 3: Identification of *Fusarium* isolates based on morphological analysis, PCR assays and sequencing of the *TEF1-α* gene region

Isolate	Location	Sample number/Method	Host	PCR assays		TEF1-α identification (%a)	Identity attribute
				ITS1, ITS4	EF1, EF2		
IN 2	Alborz	2/FM	Corn	+	+	*F. verticillioides* (99%)[b]	*F. verticillioides*
IN 3	Alborz	2/FM	Corn	+	+	*F. verticillioides* (100%)[b]	*F. verticillioides*
IN 4	Tehran	3/FM	Corn	+	+	*F. proliferatum* (99%)	*F. proliferatum*
IN 6	Alborz	4/FM	Corn	+	+	*F. verticillioides* (100%)	*F. verticillioides*
IN 7	Alborz	4/FM	Corn	+	+	*F. proliferatum* (99%)[c]	*F. proliferatum*
IN 8	Alborz	5/FM	Corn	+	+	*F. verticillioides* (100%)[b]	*F. verticillioides*
IN 9	Tehran	6/FM	Corn	+	+	*F. verticillioides* (99%)[b]	*F. verticillioides*
IN 12	Tehran	13/FM	Feed	+	+	*F. proliferatum* (100%)[c]	*F. proliferatum*
IN 14	Qazvin	14/FM	Corn	+	+	*F. proliferatum* (100%)[c]	*F. proliferatum*

IN 15	Qazvin	19/FM	Corn	+	+	*F. verticillioides* (100%)[b]	*F. verticillioides*
IN 17	Qazvin	19/FM	Feed	+	+	*F. verticillioides* (99%)[b]	*F. verticillioides*
IN 19	Tehran	21/FM	Feed	+	+	*F. verticillioides* (100%)[b]	*F. verticillioides*
IN 20	Qom	22/FM	Corn	+	+	*F. verticillioides* (99%)[b]	*F. verticillioides*
IN 23	Qom	24/FM	Corn	+	+	*F. verticillioides* (100%)[b]	*F. verticillioides*
IN 24	Qom	24/FM	Corn	+	+	*F. proliferatum* (100%)[c]	*F. proliferatum*
IN 25	Qom	25/FM	Corn	+	+	*F. verticillioides* (100%)[b]	*F. verticillioides*
IN 26	Qom	26/FM	Corn	+	+	*F. verticillioides* (99%)[b]	*F. verticillioides*
IN 27	Qom	26/FM	Corn	+	+	*F. verticillioides* (100%)[b]	*F. verticillioides*
IN 28	Qom	26/FM	Corn	+	+	*F. proliferatum* (100%)[c]	*F. proliferatum*
IN 32	Qom	27/FM	Corn	+	+	*F. proliferatum* (99%)[c]	*F. proliferatum*
IN 33	Alborz	30/FM	Corn	+	+	*F. verticillioides* (100%)[b]	*F. verticillioides*
IN 36	Alborz	30/FM	Corn	+	+	*F. verticillioides* (99%)[b]	*F. verticillioides*
IN 37	Alborz	30/FM	Corn	+	+	*F. verticillioides* (99%)[b]	*F. verticillioides*
IN 38	Tehran	31/FM	Corn	+	+	*F. proliferatum* (100%)[c]	*F. proliferatum*
IN 40	Tehran	31/FM	Corn	+	+	*F. verticillioides* (100%)[b]	*F. verticillioides*
IN 42	Qazvin	32/FM	Corn	+	+	*F. proliferatum* (99%)[c]	*F. proliferatum*
IN 44	Alborz	33/FM	Corn	+	+	F. verticillioides (100%)[b]	*F. verticillioides*
IN 45	Alborz	33/FM	Corn	+	+	F. proliferatum (100%)[c]	*F. proliferatum*
IN 46	Alborz	34/FM	Corn	+	+	F. verticillioides (99%)[b]	*F. verticillioides*
IN 47	Tehran	36/FM	Corn	+	+	F. proliferatum (99%)[c]	*F. proliferatum*
IN 49	Qazvin	37/FM	Corn	+	+	F. verticillioides (100%)[b]	*F. verticillioides*
IN 50	Qazvin	37/FM	Corn	+	+	F. verticillioides (99%)[b]	*F. verticillioides*
IN 52	Qazvin	39/FM	Corn	+	+	F. verticillioides (100%)[b]	*F. verticillioides*
IN 53	Qazvin	39/FM	Corn	+	+	F. proliferatum (100%)[c]	*F. proliferatum*
IN 54	Qom	42/FM	Feed	+	+	F. verticillioides (99%)[b]	*F. verticillioides*
IN 55	Qom	42/FM	Feed	+	+	F. proliferatum (99%)[c]	*F. proliferatum*
IN 58	Alborz	54/FM	Corn	+	+	F. proliferatum (100%)[c]	*F. proliferatum*
IN 59	Alborz	54/FM	Feed	+	+	F. verticillioides (100%)[b]	*F. verticillioides*
IN 60	Alborz	54/FM	Feed	+	+	F. verticillioides (99%)[b]	*F. verticillioides*
IN 61	Alborz	54/FB	Feed	+	+	F. verticillioides (100%)[b]	*F. verticillioides*
IN 62	Alborz	55/FM	Feed	+	+	*F. verticillioides* (99%)[b]	*F. verticillioides*
IN 63	Alborz	55/FM	Feed	+	+	*F. verticillioides* (99%)[b]	*F. verticillioides*
IN 64	Alborz	57/FM	Feed	+	+	*F. proliferatum* (100%)[c]	*F. proliferatum*
IN 65	Alborz	57/FM	Feed	+	+	*F. verticillioides* (100%)[b]	*F. verticillioides*
IN 66	Alborz	57/FM	Feed	+	+	*F. verticillioides* (99%)[b]	*F. verticillioides*
IN 67	Alborz	57/FM	Feed	+	+	*F. proliferatum* (100%)[c]	*F. proliferatum*
IN 68	Qom	57/FB	Feed	+	+	*F. verticillioides* (100%)[b]	*F. verticillioides*
IN 70	Qazvin	58/FM	Feed	+	+	*F. verticillioides* (99%)[b]	*F. verticillioides*
IN 71	Alborz	58/FM	Feed	+	+	*F. verticillioides* (100%)[b]	*F. verticillioides*
IN 72	Alborz	58/FB	Feed	+	+	*F. verticillioides* (100%)[b]	*F. verticillioides*
IN 74	Markazi	70/FM	Feed	+	+	*F. verticillioides* (100%)[b]	*F. verticillioides*

IN 75	Markazi	70/FM	Feed	+	+	*F. verticillioides* (100%)[b]	*F. verticillioides*
IN 78	Markazi	71/FB	Feed	+	+	*F. proliferatum* (99%)[c]	*F. proliferatum*
IN 79	Qazvin	65/FM	Feed	+	+	*F. verticillioides* (100%)[b]	*F. verticillioides*

a. Percentage of maximum identity of TEF1-a sequences based on Blast database. Identity of TEF1-a sequences according to Blast database; b. MF803750.1; c. MH425306.1

Based on frequency as well as relative density, *F. verticillioides* was the predominant *Fusarium* species (13.4% of the total isolations and 68.5% of the *Fusarium* isolates) with the highest incidence in Qazvin (22.5%) and the lowest in Markazi (6.8%). Nonetheless, the incidence of *F. proliferatum* was 3.4% in Qom and 4.5% in Markazi.

The incidence of *F. verticillioides* in Markazi was significantly higher ($p<0.05$) than in Tehran and Qom. Also, the incidence of *F. verticillioides* in Qazvin was significantly higher ($p<0.05$) than in Qom and Alborz, no significant difference was found between Markazi and Qazvin, Markazi and Alborz, Tehran and Qazvin, and Tehran and Alborz ($p>0.05$).

The high frequency of *F. verticillioides* in Mazandaran is the reverse to that cited by Norred [35], namely that the incidence of *F. moniliforme* infection in the high esophageal cancer region of Iran was low in 1982. In contrast, the high incidence of *F. verticillioides* in Mazandaran supports the reports on high levels of fumonisins in maize from this province [36]. The mean incidence of *F. verticillioides* was 21.4% to 59.0% in corn from major production locations in Iran [2].

CONCLUSION

Our research demonstrated that feed and corn were contaminated by hazardous amounts of fungi genera (especially potentially mycotoxigenic fungi belonging to *Penicillium*, *Fusarium* and *Aspergillus* species) higher than the principles limits. So, it is necessary to perform constant assessments of the mycological status of feed. Moreover, it is recommended to perform usual decontamination feed and corn with fungal growth inhibitors (especially against the *Fusarium* species such as *F. verticillioides* and *F. proliferatum*). Also, we advise examining the seeds before the use in agriculture to decrease the level of external and especially internal mycoflora of feed ingredients using simple techniques such as Freeze Blotter method.

The ethical code for this research is 91002395 recorded in following link: research2.bmsu.ac.ir/general/homePage.action.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this paper.

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