Original Article

Isolation and Molecular Identification of *Deoxynivalenol*- and *Fumonisin*-producing Genes from Maize Feed Contaminated with *Fusarium* Fungus in Silos of Dairy Farms in Fars province, Iran

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ABSTRACT

Mycotoxins are toxins produced by various types of fungi, including *Fusarium*, which can produce different types of mycotoxins, such as Deoxynivalenol (DON), Zearalenone, T-2 toxin, and Fumonisins (FUM). Mycotoxins have the potential to reduce the quality of crops and pose health risks to both humans and animals. This can result in reduced animal production and substantial economic consequences on a global scale. Extensive research has been carried out to investigate the high incidence of contamination in grains by *Fusarium* fungi. In this study, 80 samples of maize from silos of dairy farms in Fars province, Iran were collected and examined for fungal contamination by *Fusarium* and potential production of DON and FUM. For this purpose, identification using specific primers for different genes was carried out by polymerase chain reaction test and gel electrophoresis with agarose (1%). Among different counties, the silos in Kharameh with 47.05% and Jahrom with 46.15% had the highest contamination rates, and the lowest contamination rate belonged to Kazeroon with 27.27%. Out of the 30 positive samples contaminated by *Fusarium*, 21 produced FUM, 4 produced DON, and 5 produced both toxins. *Fusarium* species that contaminated the maize samples were also identified, including 13, 10, and 7 samples contaminated by *F. proliferatum*, *F. verticillioides*, and *F. graminearum*, respectively. As a conclusion, the findings of the study indicated that 37.5% of the corn samples from dairy farm silos in Fars province were contaminated with the *Fusarium* fungus, which had the potential to produce the toxic mycotoxins, deoxynivalenol, and fumonisins.

Keywords: *Deoxynivalenol, Fumonisin, Fungi, Fusarium, Mycotoxin*

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1. Introduction

The term "mycotoxin" originates from the Greek words "*mykes*", meaning fungus, and "*toxikon*", meaning poisonous spear (1). Mycotoxins are secondary metabolites produced by fungi that can contaminate agricultural products such as corn, wheat, and other crops. Contamination of products may occur at various stages, including cultivation, harvest, storage, transportation, and distribution of the products (2). Globally, the safety and quality of agricultural commodities are major concerns that frequently jeopardized by mycotoxin contamination in various feedstuffs (3). The existence of mycotoxins can adversely impact the quality of crops, as well as the health of both humans and animals, ultimately leading to a decline in animal production and significant economic impacts worldwide (4-6). The first documented case of food poisoning with mycotoxins was likely ergotism in 1853. Ergotism caused limb necrosis, referred as the "fire of God" in the middle Ages in Europe. The disease resulted from consuming grain contaminated with the fungus *Claviceps purpurea*. Another fungal poisoning accompanied by an epidemic in the human population occurred in 1953, termed toxic food leukopenia (7). Symptoms in humans varied and included food poisoning, bloody diarrhea, leukopenia, necrotic lesions in the oral cavity, esophagus, and stomach, brain, and bone discharge (4). A post-World War II report from Japan highlighted food poisoning caused by consuming yellow rice imported from different countries. Consumption of yellow rice induced nausea, convulsions, and progressive paralysis, leading to death within 1 to 3 days of symptoms onset. The fungus Penicillium was identified as the toxin-producing agent in rice (8). *Fusarium* is one of the economically important genera of phytopathogenic fungi known to infect cereal crops such as maize, wheat, barley, oat, rice, and millet with toxigenic species. Apart from causing plant diseases, *Fusarium*'s secondary metabolites, including fumonisins (FUM), deoxynivalenol (DON), zearalenone (ZEN), T-2/HT-2 and similar metabolites, raise public health concerns due to their toxicity. The transmission of mycotoxins from feed-to-animal products, such as milk and meat, pose significant risks to humans. The European Union has issued guidelines for specific mycotoxins in cereal feed, including 2, 8, 60, and 0.5 mg/kg for ZEN, DON, FUM, and T-2/HT-2, respectively, for all cereals except for maize, for which the guidance levels for ZEN and DON are 3 and 12 mg/kg, respectively (9). *Fusarium*'s mycotoxins are a major concern in crop production due to their impact on crop quality, animal production, and public health. Deoxynivalenol, zearalenone, and fumonisins are among the most prevalent *Fusarium* mycotoxins in agricultural commodities. Their biosynthesis is regulated by environmental factors, including temperature, humidity, exposure to oxygen, crop damage, and mold spore contamination (10, 11). DON is typically produced by *F. graminearum*, and *F. culmorum* and found in various cereal commodities, including wheat, maize, barley, and oats (12). The stability of DON, even under high-temperature treatments during food processing, renders it challenging to mitigate in contaminated foods (13). FUM are primarily produced by *F. verticillioides* and *F. proliferatum* and commonly contaminate maize crops globally. The toxic FUM group includes three types known for their considerable toxicity: FB_1, FB_2, and FB_3. Among these, FB_1 has been designated by the International Agency for Research on Cancer (IARC) as a potential human carcinogen belonging to Group 2b (14). Additionally, FUM toxins have been found to be neurotoxic, influencing the biosynthesis pathway of sphingosine, which is fundamentally essential for the brain and nervous system. Based on the reports of the Food and Agriculture Organization of the United Nations (FAO), millions of tons of food products are lost annually due to mycotoxin contamination. Therefore, since 1988, numerous programs, along with training workshops, have been implemented globally to control food hygiene, including milk,. According to a report by the Centers for Disease Control and Prevention, microorganisms and pathogenic fungi have been the cause of many cases of food poisoning in the United States (15). The present research aims to explore the prevalence of *Fusarium* fungi contamination in maize silos on multiple dairy farms across the Fars province, and also examining the frequency of DON and FUM producing genes in the isolated samples to evaluate the potential risk of mycotoxin contamination in the local agricultural commodities.

2. Materials and Methods

This study was a cross-sectional investigation conducted over three months, from March to June 2022. Maize silos from dairy farms located in Fars province, Iran, were randomly sampled, resulting in a total of 80 samples. The silo samples were directly collected and stored away from moisture. Approximately 100 grams of each sample were placed in clean and sterile plastic bags and transported to the laboratory. The samples underwent initial culturing on dextrose agar (Merck-Germany), and *Fusarium* fungus was isolated and purified using CLA, SNA, and ZDB media (Merck-Germany).

2.1. Isolation of Fungi

In order to isolate fungi, 30 g of the harvested samples were milled, and 1 g of each sample was mixed with 10 ml of sterile distilled water. After allowing it to stand for 1 hour, 100 µL of the supernatant was cultured on SDA

Agar (Merck-Germany) using a sterile swab. Subsequently, the sediment was also cultured on SDA plates (16) after discarding the supernatant. To determine the superficial fungal flora, 10 grains of maize were cultured on SDA medium with chloramphenicol antibiotic, excluding saprophytic fungi. The obtained isolates were purified by culturing samples on PDA (Merck-Germany) medium and storing them at 25°C. A 4 mm mycelium block was then transferred from the margin of each colony to a test tube containing distilled water and shaken. The suspension was poured on the PDA medium to obtain pure isolates. The culture media were incubated at 25°C under a hood with a half-open lid for 30 minutes and then transferred to the incubator. The single colonies were transferred as a pure culture to a new PDA medium, and the isolated fungi were identified based on morphological characteristics, including colony color, hyphae features, type of symbiotic organ, and the shape and color of spores (17).

2.2. DNA Extraction and PCR Process

DNA extraction was performed using the phenol-chloroform method. Mycelium pieces were mixed in lysis buffer, and grinding was done using an electric herb grain grinder. The supernatant was transferred to a new tube and mixed with an equal volume of phenol-chloroform; then samples were centrifuged. The liquid phase was transferred to a new tube, and twice the volume of 2-propanol and 3.1 times of sodium acetate were added to the tube and placed at -20 °C for 24 hours. After centrifugation at 13000 rpm for 10 minutes, the supernatant was discarded. The pellete was washed using 1 mL of ethanol (70%) in order to remove salt and alcohol. Then, the sediment was dissolved in 20 µL of TE buffer and stored at -20 °C. For performing polymerase chain reaction (PCR), 5 µL of the extracted DNA, 1 µL of each of the forward and reverse primers (designed by Rasa Gene company), and 12.5 µL of master mix (dNTP, $MgCl_2$, PCR buffer, Taq polymerase) were added to a PCR tube. The final volume was adjusted to 25 µL with distilled deionized water, and the tube was centrifuged for 20 seconds at 4000 rpm before being placed in a thermal cycler. The PCR program was set with initial denaturation at 95 °C for 5 minutes followed by 38 cycles of 95 °C for 1 minute for denaturation, primer annealing at 60 °C for 1 minute, and extension at 72 °C for 1 minute, with a final extension process at 72 °C for 5 minutes. To visualize the reaction results, 5 µL of the PCR products were electrophoresed on a 1.5% agarose gel in a tank containing TBE buffer (containing boric acid, EDTA and tris). Finally, the agarose gels were placed in the gel doc device and different bands were evaluated.

3. Results

In this study, a total of 80 samples were collected from silos of cattle farms in the Fars province over a period of three months (March to June 2022). Among these samples, 30 were identified as infected with various species of *Fusarium* fungi. The 30 positive samples including 8 from Kharameh, 6 from Jahrom, 5 from Beyza, 4 from Zarqan, 4 from Sepidan, and 3 from Kazerun were identified (Table 2). Based on the ratio of positive samples to the total number of samples (N/P ratio) for *Fusarium* contamination, Kharameh had the highest infection rate at 47.05%, followed by Jahrom with 46.15%, Beyza with 38.46%, Sepidan with 33.33%, Zarqan with 28.57%, and Kazeroon with 27.27% (Figure 1).

Table 1. Characteristics of primers used in PCR

Primer type	PCR product size	Primer's sequence (5' --> 3')	references
18srRNA	561 bp	CACCAGACTTGCCCTCCA	(18)
	561 bp	AACCTGGTTGATCCTGCCAG	
FUM	1596 bp	CAGTACATGCCCCAAGCC	-
	1596 bp	CGAGAGTCGGAACTACGAGC	
DON	160 bp	AGCAGCTTCACCAGTACCAC	-
	160 bp	TCGGTGACAGCCTCAACATC	

Table 2. The number of total and positive samples collected from different counties

Province	County	Number of samples	Positive samples
Fars	Zarqan	14	4
	Jahrom	13	6
	Kazerun	11	3
	Beyza	13	5
	Sepidan	12	4
	Kharameh	17	8
Total		80	30

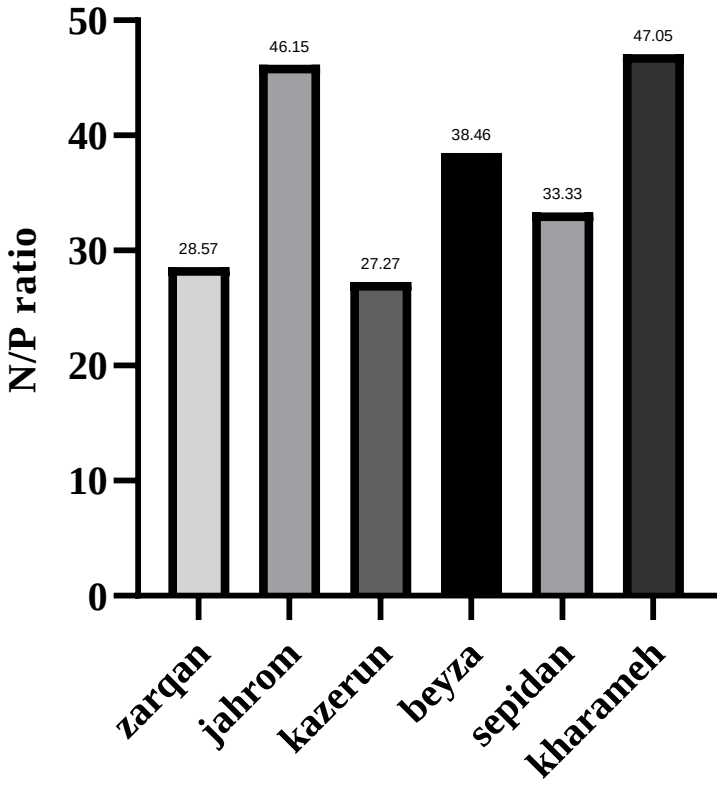

Figure 1. N/P ratio of samples collected from different counties of Fars province

Three species, including *F. graminearum*, *F. verticillioides*, and *F. proliferatum*, were identified based on morphological characteristics such as colony color, hyphal characteristics, symbiotic organ type, and spore shape and color. Out of 100 infected samples, seven cases of infection were caused by *F. graminearum*, ten cases by *F. verticillioides*, and thirteen cases by *F. proliferatum* (Table 3). The objective of this study was to investigate the presence of genes responsible for producing FUM and DON mycotoxins. After conducting PCR using specific primers (Table 1), the products were analyzed by gel electrophoresis using a 100 bp ladder as well as positive and negative controls. According to (Figure 2), eight samples were analyzed for DON (top) and FUM (bottom) toxins, and all of them were found to contain both genes.

Table 3. The type of fungus producing toxin and the genes producing toxin FUM and DON.

Total samples	Total positive samples	species	Positive samples
80	30	*F. graminearum*	7
		F. verticillioides	10
		F. proliferatum	13

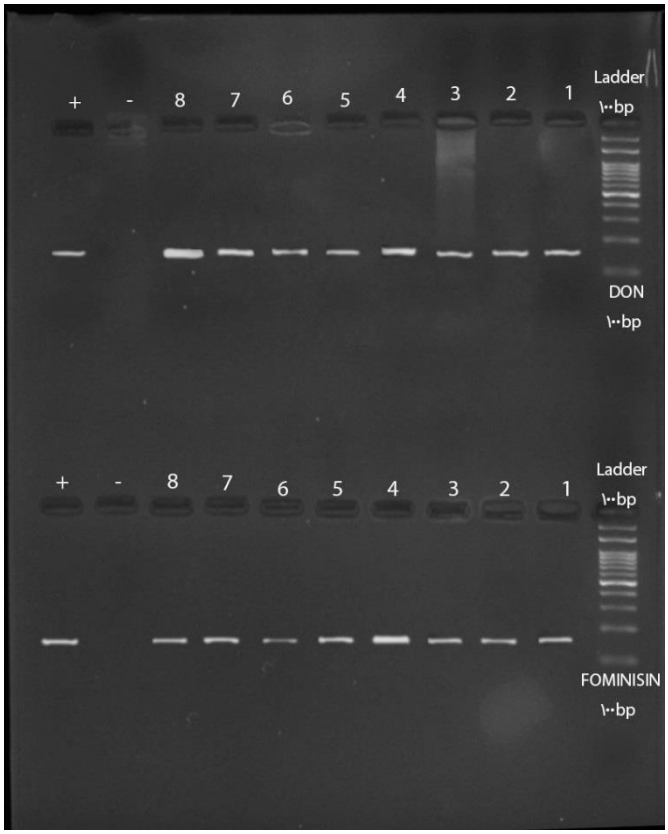

Figure 2. The molecular weight of PCR products was determined and compared to the molecular weights of DON and FUM genes

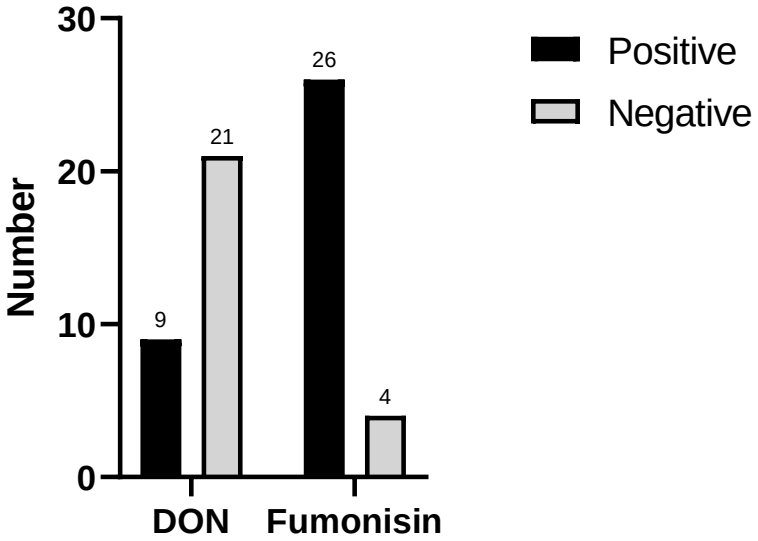

Figure 3. The number of *Fusarium*-infected samples containing genes encoding DON and FUM toxins

Molecular analysis of the samples indicated that out of 30 infected samples, nine were contaminated with *Fusarium* fungi carrying the deoxynivalenol-encoding genes, and 26 samples were contaminated with fumonisin-producing species (Figure 3). Specifically, 13.33% of the infected samples produced only FUM toxins, 70% produced only DON toxins, and 16.67% produced both FUM and DON toxins (Figure 4).

4. Discussion

Mycotoxins, toxins produced by fungal species, are known to be diverse, with a single fungus capable of producing multiple types of toxins. To date, over 400 different mycotoxins have been identified and, extensive research have been conducted on mycotoxins secreted by *Fusarium* fungi due to their significance in poisoning (19). *Fusarium* fungi exhibit the capability to infect various plants types including crops like wheat, maize, oats, rye, barley, and pastures. Main mycotoxins produced by the *Fusarium* species includes DON, t-2 toxin, ZEN, and FUM (20, 21).

The Food and Drug Administration in the United States establishes limits for mycotoxins, with recommended limits for nursery pigs set at 1 mg/kg for DON concentrations and 10 mg/kg for FUM in completed feeds (22). Similarly, the European Commission has advises an upper limit of 0.9 mg/kg for DON and 5 mg/kg for FUM (23,24). Among 80 samples obtained from corn siloes across various cattle farms in different regions of Fars province, 37.5% were found to be contaminated with *Fusarium* fungi carrying the genes for producing FUM and DON mycotoxins. Simoes et al. conducted a study from 2019 to 2020 in Tagus Valley, Portugal, examining 1650 grain samples. Of these, 344 were found to be infected with *Fusarium* fungi. Among the infected samples, 117 were found in maize, with 69.2%, 29.1% and 1.7% of them infected with *F. verticillioides*, *F. subglutinans*, and *F. proliferatum*, respectively. In the current study, the prevalence of these species was 43.33%, 33.33%, and 23.33% for *F. proliferatum*, *F. verticillioides*, and *F. graminearum*, respectively. Comparing the Simoes et al.

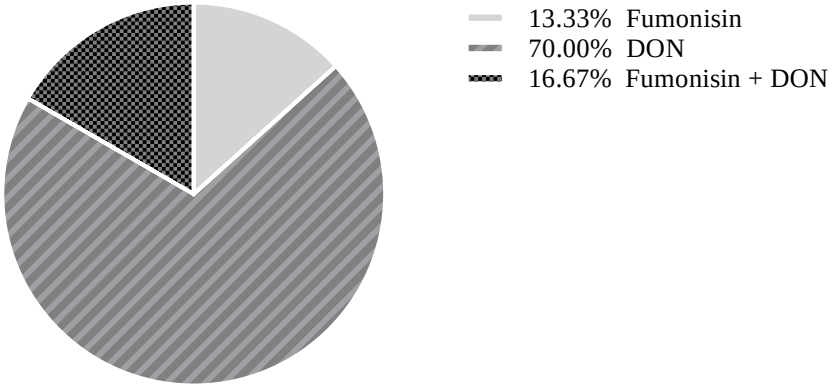

Figure 4. The frequency of DON and FUM encoding genes in maize samples from silos of cattle farms.

study (25) the prevalence of *F. verticillioides* was lower, and *F. proliferatum* was higher in the current study. Out of 80 samples from silos in Fars province, Iran, 30 were found to be infected with *Fusarium* fungi. In comparison, Topi et al. study in Albania reported infection rates of 84% in the first year and 57% in the second year. The differences in species and toxin type are influenced by changes in environmental conditions, making such variations natural. The contamination rate of samples with DON was 30% in the current study and 24% in Topi et al. study, while for FUM, both studies reported contamination rates of 86.7% and 76%, respectively. This showed that the species producing FUM were more prevalent in both studies than those producing DON, considering the highly similar results in both studies (26). Castanares et al. conducted a study on *Fusarium* species affecting maize in Buenos Aires, Argentina, where *Fusarium* verticillioides made up 81.2% of the identified *Fusarium* species over three years, followed by *F. subglutinans*, *F. graminearum*, *F. proliferatum*, and *F. cerealis* at 9.07%, 9.25%, 0.38%, and 0.1%, respectively. About 90% of these species produced DON, and the production of FUM ranged from 0 to 3%. In contrast, the present study found a higher production rate of FUM than DON, and *F. proliferatum* was the dominant contaminating species (27). Considering that environmental conditions including temperature and water activity have a significant impact on the growth and toxin production of different *Fusarium* species, it is irrefutable for various studies to have differences in their results (28).

Given the variability in the type and quantity of toxins produced by *Fusarium* strains contaminating animal feed due to environmental changes, this study focused on silage corn samples obtained from cattle farms in Fars Province to investigate the types of contaminating microorganisms and the produced toxins (29,30). The study aims to offer more precise and rapid guidance for preventive measures in the event of animal feed contamination with *Fusarium* fungi, including their various types of toxins, an aspect not explored in prior research. In order to enhance the accuracy of these studies, additional research should be conducted in each region to identify the contaminating species and their produced toxins with higher precision. This approach allows for more targeted measures to prevent the financial and health risks associated with *Fusarium* contamination of feed. Although the present study had limitations, such as not measuring the amount of toxin produced in each product and the short duration of the study. Furthermore, other *Fusarium* toxins, including ZEN, were not investigated in this study. Therefore, further studies should consider these factors. These findings highlights the presence of *Fusarium* species in cattle farms silos in the Fars province, Iran, and their ability to produce mycotoxins, posing a significant risk to human and animal health. Three species of *F. proliferatum*, *F. verticillioides*, and *F. graminearum*, contaminated the maize samples, with 21 cases producing fumonisins, 4 cases produced deoxynivalenol, and 5 cases produced both toxins. The results emphasize the necessity for regular monitoring of *Fusarium* species and mycotoxin contamination in agricultural products to prevent the potential harm associated with these harmful fungi and substances.

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Authors' Contribution
Study concept and design: Taghinejad J, Zakikhani F.
Acquisition of data: Afshar S. Fattahi H.
Analysis and interpretation of data: Taghinejad J, Afshar S.
Drafting of the manuscript: Taghinejad J, Afshar S.
Critical revision of the manuscript: Fattahi H. Zakikhani F.
Statistical analysis: Taghinejad J, Afshar S.

Ethics
The authors have observed all ethical points including non-plagiarism, double publication, data distortion and data manipulation in this article.

Conflict of Interest
The authors declare that they have no conflicts of interest.

Data Availability
The data that support the findings of this study are available on request from the corresponding author.

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