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Molecular identification of Aflatoxin B1 *Aspergillus flavus* in red, black and white pepper using PCR method

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ABSTRACT

In this study, the prevalence of potential *Aspergillus flavus* producing aflatoxin B1 in three different types of pepper in Tehran grocery has been investigated. The experiments were performed on 90 samples of pepper, including 30 black pepper, 30 white pepper and 30 chilli peppers, and cultivated on Sabouraud dextrose agar. The isolation of the genus and species of fungi was performed by macroscopy and microscopy examination. In order to confirmation of *A. flavus* diagnosis, polymerase chain reaction (PCR) was performed using specific species primers. The ability to produce aflatoxin B1 was determined by specific primers to detect four genes from the aflatoxin B1 biosynthesis pathway. The results showed that the isolated fungi of red pepper samples consisted of 93.3% of *Mucor* species, 16.6% of *Aspergillus niger* and 3.3% of *A. flavus*. Also isolated fungi from black pepper samples were 36.6% of *Mucor* species, 33.3% of *A. flavus* and 30% of *A. niger*. The results on white pepper represented 40% of *Mucor* species, 36.6% of *A. niger* and 33.3% of *A. flavus*. The results of PCR showed that all *A. flavus* possesses *nor-1*, *ver-1*, *omt-1* and *afR1* genes and potentially produced aflatoxin B1. Only one sample of white pepper and one sample of black pepper lacked the *omt-1* and *aflR* gene, which are therefore unable to produce aflatoxin. The presence of toxigenic fungi such as *A. flavus* in pepper can produce mycotoxin that affects the quality of pepper and causes human disorder.

1. Introduction

Spice is called additive and food stuffing, which improves the taste and taste of the food. Since human health is dependent on the health of the food, pepper, like any other food, needs to be healthy due to its high consumption (Balarman et al., 1997; Hitokoto et al., 1980). Pepper is susceptible to contamination with microbial contaminants such as toxins of some fungi. Scientists After years of research, the 1961 aflatoxin producing fungus eventually named *Aspergillus* (Carrajal et al., 2003). *Aspergillus* is a group of fungi that, due to

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aflatoxin contamination, endanger human and animal life. There are about 900 species of *Aspergillus* species, all of which are present in the environment and are grown in soil, on vegetables, in organic matter, in corrosive material, in food debris, in medicines and so on (Colack et al., 2006; Dieckman et al., 1992). *A. flavus* and *A. parasiticus* are the most important producers of aflatoxin (Nazari et al., 2014). Aflatoxin B1 is the strongest mutagenic and carcinogenic agent among fungal toxins. The main symptoms of aflatoxicosis is the decreased growth and weakening of the immune system (Yentur et al., 2012). Acute aflatoxicosis occurs in humans following the consumption of contaminated food, as chronic hepatitis with symptoms such as jaundice, diarrhea and fatty tissue degradation (Park et al., 2005). Chronic aflatoxicosis is a causative agent of liver cancer and its main mechanism is the mutation in the P53 tumor suppressor genes. Human is directly affected by aflatoxin contaminated food (Cotty et al., 2007). This poison is a natural fungal toxin that originates from *A. flavus* and *A. parasiticus*. *Aspergillus*, in particular, three species of *A. flavus*, *A. parasiticus* and *Aspergillus nomius*, are from aflatoxin producing fungi (Geeta et al., 2007). This fungus is one of the ascomycetes, their spores are scattered in the air. According to studies on pepper and the report on the presence of aflatoxin producing fungi, studies are needed to investigate the presence of fungi and their metabolites in pepper, until in the event of contamination, measures should be taken to control fungal growth and production of aflatoxins (Mekawey et al., 2019; Kim et al., 2019). According to the mentioned issues and the probability of fungal contamination, the aim of this study was to evaluate the prevalence of Aflatoxin B1 potential asparagilosis in three types of black, white and red pepper. Since today, PCR technique is one of the fastest and most specific method for determining the identity of fungi. After the cultivation of samples, the ability to produce aflatoxin B1 by fungi has been investigated using this technique.

2. Materials and Methods

2.1. Sample collection

In this study, 90 samples of pepper, including red, black and white pepper, were collected from grocery throughout Tehran regions for three months (Autumn, 2018). Samples from four regions of North, South, East and West of Tehran, each region containing many grocery, were purchased completely randomly using random numbers from each region. Of these 90 samples, 30 samples of chillies, 30 samples of black pepper and 30 samples of white pepper. Red peppers and black pepper in powder form and white pepper were in the form of very tiny white grains that were powdered.

2.2. Cultivation of pepper samples

Black pepper, white and red are completely powdered. These samples were numbered 1 to 30 for red pepper, from 1 to 30 for black pepper, and from 1 to 30 for white pepper, respectively. These numbers were completely random. To each of the test tubes containing 9 ml distilled sterilized water, one gram of sample of pepper was added. Pepper in water was well solved with the help of the vortex system, and uniformity suspension was achieved. 100 and 500 µL prepared supernatant suspension was picked up. All plates were incubated at 25°C. Plates were examined after 24 to 48 hours. The cultivation process is repeated twice.

2.3. Morphological Identification of Isolates

The fungal colonies grown on sabouraud dextrose agar medium (Merk, Germany) were examined macroscopically and microscopically. First, macroscopic color and colony form for the separation of *A. flavus* from other fungi were examined. Microscopic examination was performed by observing the specific structures of *Aspergillus* genus. In this study, below the laboratory hood, a drop of lactophenol catene blue (Micromedia, Hungary) placed on the slide. Under the sterile conditions, part of mycelium colonies of fungus from the plate was separated and placed on slide. Then observed by microscope.

2.4. Isolation of fungal colonies from the environment for DNA extraction

Pure culture was prepared to isolate the fungal colonies from the medium to extract DNA from platelets containing *A. flavus* fungi. Colonies cultivated on Sabouraud dextrose agar

and incubated. Before the production of pigment, colonies were exited from the environment. The presence of a pigmentation disrupts the DNA extraction process. Therefore, the temperature of the incubator was reduced (28 °C) and placed within it for 24 hours.

2.5. DNA extraction

To DNA extraction, at first, some amount of fugus from pure culture were taken and cuted with scalpel into smaller pieces. Then it was powdered by pouring liquid nitrogen onto it. Then this powder was poured into micro tube and used for cellular degradation and extraction of cellular DNA from the Vivantix brand kit for the extraction of DNA fungus (GF-1plant DNA extraction kit).

2.6. Confirmation of flavus Species by PCR

Specific primers of flavus species were used to perform the PCR reaction to detect the species. Primers and Mastermix 2X were prepared from Sinagen. To perform PCR and detection the ability to produce aflatoxin B1 by isolated *A. flavus* four primers of nor-1, ver-1, omt and aflR were used. These primers were specific for the biosynthesis genes of aflatoxin B1.

2.7. PCR test to check the aflatoxygenicity of A. flavus

To perform the PCR, the original solution was first prepared, known as PCR master mix. The required amount was provided in Table 1.

Table 1. Materials required for the preparation of a PCR reaction mixture

PCR materials	The amount of material in µL for one sample
Master Mix 2X PCR	10
Distilled water	5
F Primer	1
R Primer	1
DNA sample	3
Total volume	20

3. Results

3.1. Cultured samples

In macroscopic examination, green colonies were observed. Results of culturing samples of various types of fungi in three types of pepper are shown in Fig 1.

The statistical analysis in Table 2 shows the percentage of contamination in 30 samples of red, black and white pepper, broken down to each of the types of observed fungi.

3.2. performing PCR to detect flavus species

In Fig. 2, the PCR results can be observed for the diagnosis of *A. flavus* species. In Fig. 2a and b, the product was obtained from the *A. flavus* Specific Primer (FLA).

3.3. PCR to detect the aflatoxygenogenicity of the isolates

The results of identifying the effective genes in aflatoxin production in *A. flavus* are shown in Fig. 3.

Table 2. Percentage of contamination in 30 samples of different types of peppers

	Types of fungus	Percentage
Red pepper	*A.flavus*	3.3%
	Mucor	93.3%
	A. niger	16.6%
Black pepper	*A.flavus*	3.3%
	Mucor	36.6%
	A. niger	30%
White pepper	*A.flavus*	33.3%
	Mucor	40%
	A. niger	36.6%

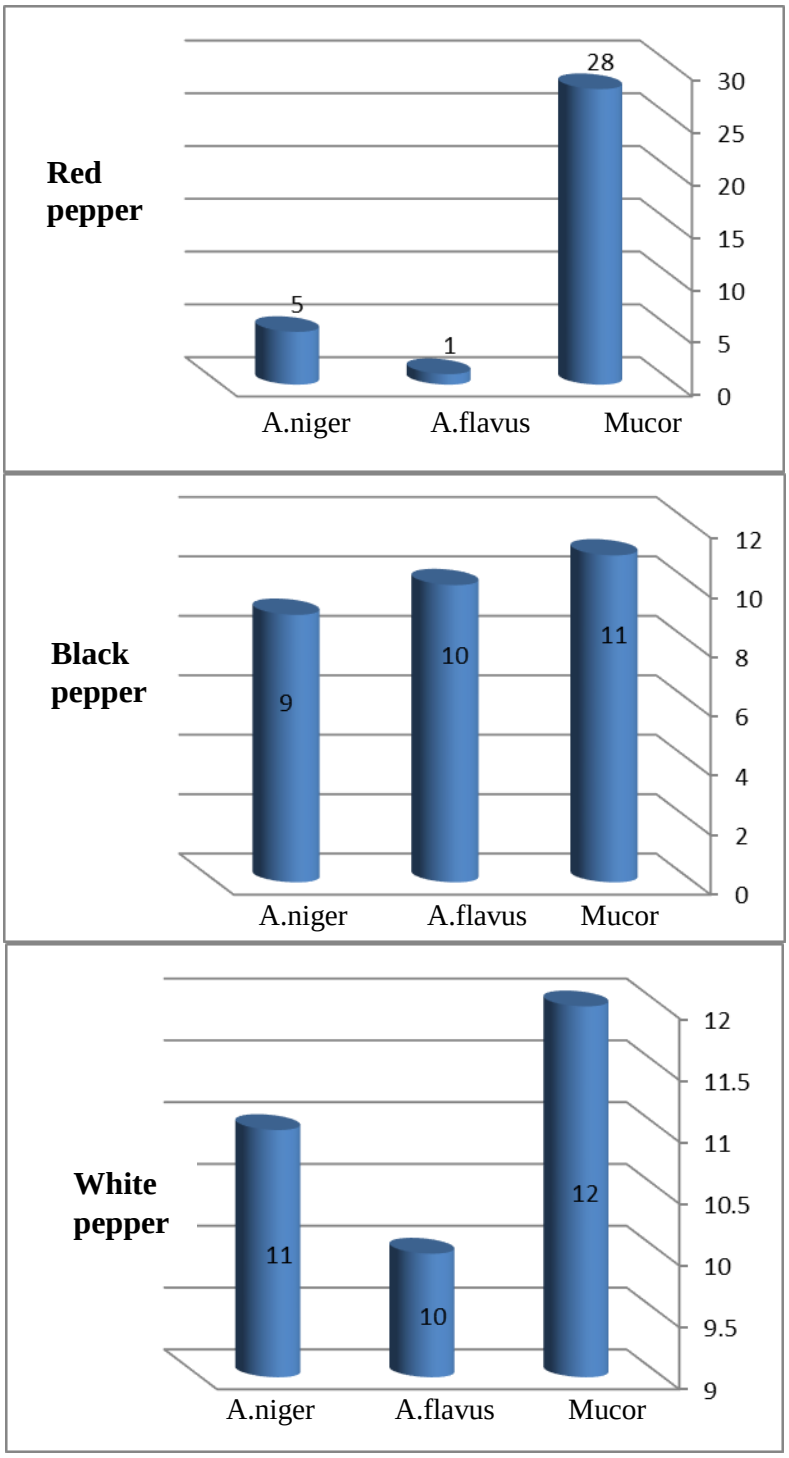
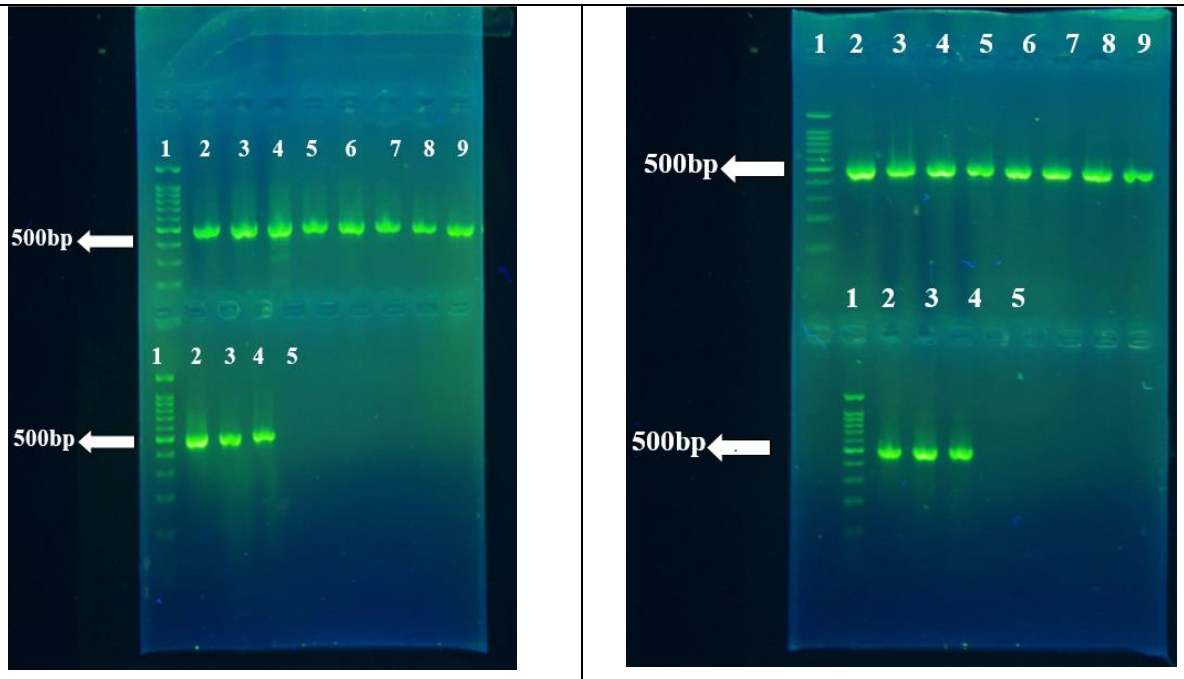
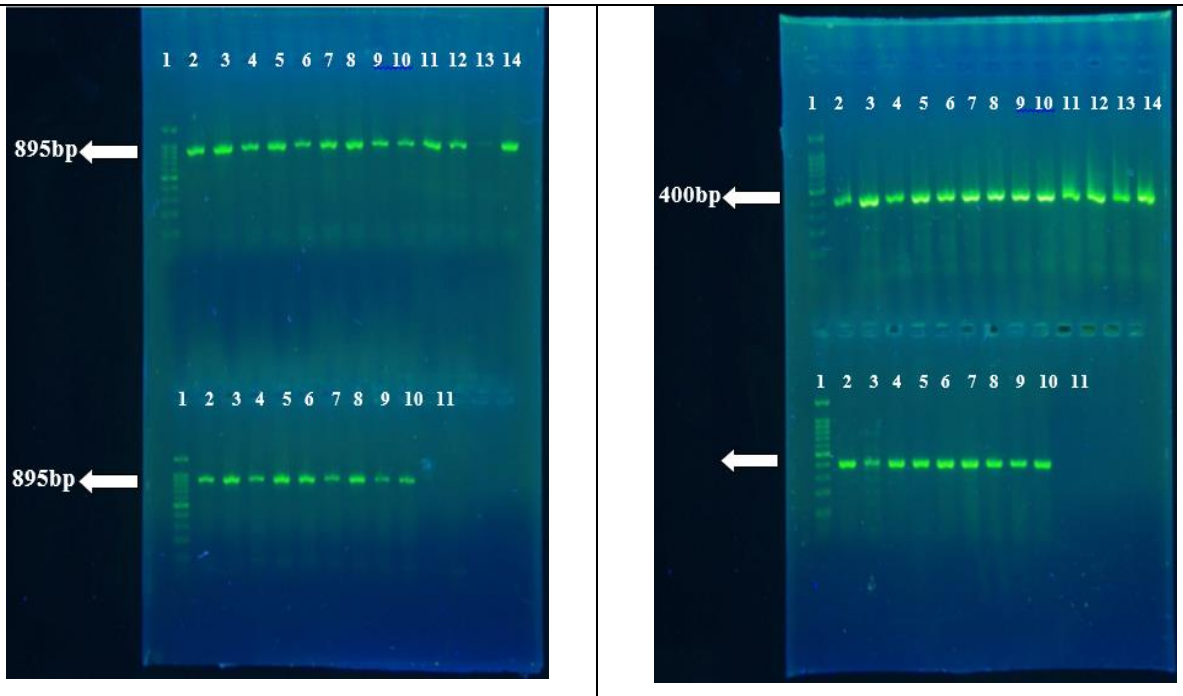


Figure 1. Frequency chart of fungi in different types of peppers

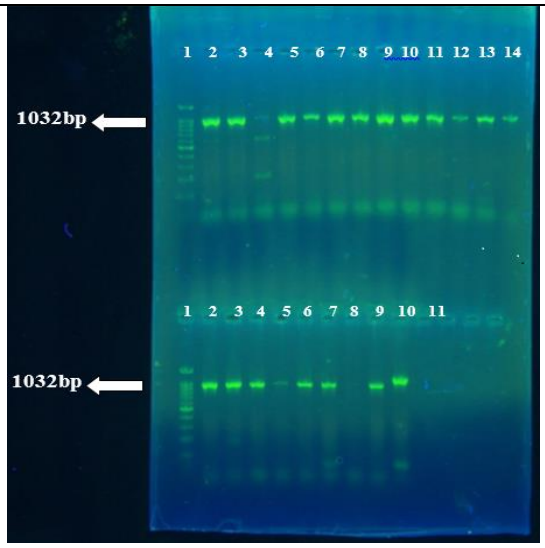


A B

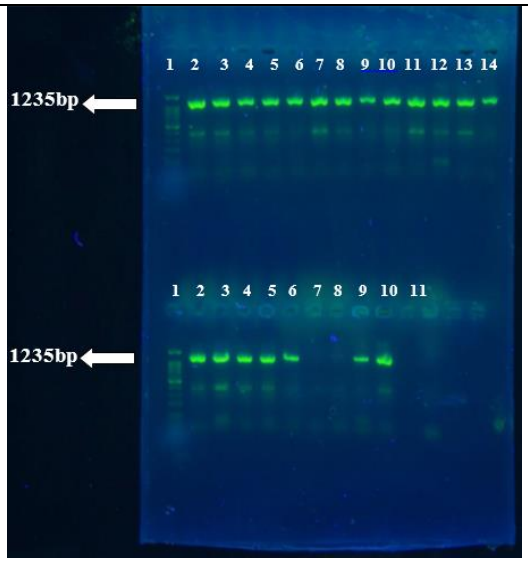
Fig 2. PCR product electrophoresis to confirm the detection of *A. flavus* from various types of peppers. The top and below lines, well 1: ladder, 2: Positive control, 3 to 9: Samples.



nor-1 ver-1



aflR



omt-1

Fig. 3 Electrophoresis of PCR product obtained with ver-1, nor-1, omt-1, and afIR primers for detecting the presence of ver-1, nor-1, omt-1 and afIR, respectively genes, and to identify the potential of aflatoxin B1 production in species Aspergillus flavius isolated from various types of peppers.

In ver-1 electrophoresis, the top of line of the well number 1: the ladder, 2: positive control, 3 to 14: samples, in the bottom line of the well No. 1: ladder, 2 to 10: samples and 11: negative control.

In the nor-1 electrophoresis, the top line of the well number 1: the ladder, 2: positive control, 3 to 14: samples in the bottom row of the well No. 1: ladder, 2 to 10: samples and 11: negative control.

Also, in the omt-1 electrophoresis, the top line of the well number 1: for the lead, 2: positive control, 3 to 14: samples in the bottom line of the well number 1: lead, 2 to 10: samples and 11: negative control.

In afIR electrophoresis, the top line of the well number 1: for the ladder, 2: positive control, 3 to 14: samples in the bottom row of the well No. 1: ladder, 2 to 10: samples and 11: negative control.

4. Discussion

Mycotoxins are biological agents that affect the production and quality of the toxic fungi in foods. Therefore, in order to ensure the health of consumers, it is necessary that the presence of fungi producing these dangerous toxins is permanently identified and minimized in the food chain (Chang et al., 2007). The dangers of aflatoxin have been proven to humans in terms of food consumption. Its dangers in human health, especially liver cancer, are indicative of raising the quality of AFB1-free food (Abdel-Hadi et al., 2011). Saadullah et al investigated that despite the *A. flavus* was grown in all samples, AFB1 was not produced by this *A. flavus* in some samples (Saadullah et al., 2014). Shundo et al found *A. flavus* were growing on all samples of black pepper and aflatoxin B1 infected about 60 µg of black pepper (Shundo et al., 2009). Ardic et al reported an enzyme-linked immunosorbent assay (ELISA) in 96% of Chilean infections with Aflatoxin B1 (Ardic et al., 2008). The results of this study in compare to our study, did not related to the frequency of contamination of red pepper samples with aflatoxin B1, which can be explained by the difference in the test method or the collection of red pepper samples from two different countries. Roze et al investigated the contamination of fungi in some imported spices, reported that the red and black pepper had the highest percentage of fungal contamination and contaminated with *Aspergillus*, *Penicillium*, *Trichotichium*, *Rhizopus* and *Chladosporium* (Roze et al., 2013). The results of this study were similar to the results of the present study on the frequency of *Aspergillus* fungi as the most isolated fungal species. Our results have been consistent with domestic and foreign research in this regard. In all of these studies, the presence of *A. flavus* in red, black and white pepper has been proven. Certainly, it can be said that in pepper specimens there is a possibility of contamination

with *A. flavus* toxic fungi. But the difference between this mentioned research with our research has been that most of these studies have achieved the dose of Aflatoxin for PP, that is, indirectly, the presence of *A. flavus* can be found in the samples. Also results of PCR showed that all *A. flavus* possesses *nor-1*, *ver-1*, *omt-1* and *afR1* genes and potentially produce aflatoxin B1.

Conclusion

The presence of toxigenic fungi such as *A. flavus* in pepper can produce mycotoxin that affects the quality of pepper and causes human disorder.

Refereces

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