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MOLECULAR DETECTION OF AFLATOXIN PRODUCING FUNGI IN ASPERGILLUS ISOLATED FROM FRESH PISTACHIO NUTS IN KERMAN

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ABSTRACT

The molecular identity of aflatoxin producing aspergillus was studied in fresh pistachio in Kerman province.Diagnostic PCR method was used in this study, in the experiment, a regulatory gene called aflR in all the samples that were infected genuse aspergillus produce aflatoxin was used for confirmation of the presence of genes. Molecular results showed that 36 fresh pistachios samples that were purposefully selected, 25 samples were healthy and only 11 were contaminated. Results showed that from infected samples, fungal infections contained aspergillus (54.54%), Penicillium (18.18%), penicillium and aspergillus, mucor, saprophyte, each was observed 9.09% and the aspergillus produce aflatoxin regulatory gene was found only in 4 cases.

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Introduction

Fungi are a group of eukaryotic microorganisms and are categorized in two groups of mold and yeast [1,2]. Fungi are very important in various aspects including pathogenicity, molecular biology, biotechnology, biological control and production of various mycotoxins [3]. Aspergillus, which is a part of the ascomycete fungi, include saprophytic and opportunistic fungi that infect the environment, food, vegetables, fruits, open cap medicines, dialysis bags and air-conditioning devices. Until now, more than 200 aspergillus species have been identified, which about 20 of them are pathogenic to human. Among the Aspergillus species, the *Aspergillus flavus* is able to produce a toxic substance called aflatoxin in certain conditions, such as in pistachios and nuts, which cause liver cirrhosis in people consuming food contaminated with this poison. Among all the discovered toxic biological substances, aflatoxins are the strongest liver carcinogen substances ever [4-6]. The production of aflatoxin and the contamination of agricultural products are the main cause of economic losses in agriculture. Infection of pistachio with aspergillus and its mycotoxins is the most important problem in the consuming and export of this product [7]. Aflatoxins are a group of mycotoxins and secondary metabolic from different species of aspergillus, usually produced by *Aspergillus flavus* and *Aspergillus parasiticus* species [8, 9]. Aflatoxins are usually found in foods such as wheat, rice, cereals, barley and agricultural products. The poisonous agent is genotoxic and has a significant role in carcinogenesis, mutagenesis, teratogenesis and has estrogenic effects on humans and animals [10, 12]. Production of aflatoxin at pre-harvest time is because of factors such as temperature, water availability, plant nutrition, weed infestation, insects, bush density, agronomic rotation, drought stress, presence of antifungal compounds and microbial contamination. Also, post-harvest factors involved in the production of this toxin include temperature, water availability, oxygen, carbon dioxide, insects, rodent infections, broken seeds, product cleaning, toxigenic fungi load, microbial competition, presence of antifungal compounds, composition of the substrate, waiting for drying and storage conditions [13]. The appropriate temperature for aflatoxin production is 25 to 40 °C and the required moisture content is 85 percent [14]. Pichia anomala yeast, a kind of ascomycete and teleomorphic mushroom of the pichia genus, is the only biological control yeast inhibiting the growth and production of aflatoxin from *Aspergillus*

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flavus [15, 16]. Recently, 2- phenylethanol has been identified as volatile compounds produced by this yeast [17]. 2-Phenylethanol is widely found in nature, especially in flower extracts and aromatic oils. In addition to this yeast, Saccharomyces cerevisiae can also produce this volatile compound [18, 19]. 2-phenylethanol decreases the growth of *Aspergillus flavus* and other fungi as well as aflatoxin production on pistachio trees [20]. Compared to other nuts, pistachios have low calorie content, the most antioxidants, no cholesterol, and a small amount of saturated fat. Fat is an energy source and helps to absorb vitamins D, E, A and k [21].

Genes enrolling as regulators of aflatoxin biosynthesis pathway, include aflR and aflS, and indeterminate genes in aflatoxin biosynthesis pathway whose role is still unclear. The polymerase chain reaction is a method in which a repetitive periodic program of warming and colding the DNA with a heat-resistant DNA polymerase and two specific DNA sequences as primers are used to selectively proliferate a specific small portion of the genome. Proliferation of DNA with this method is a logarithmic process, thus it is possible to obtain large amounts of DNA. As an example, when very small amounts of special sequences is available, PCR is used, and in order to study these genes, DNA has been multiplied frequently enough to be available in sufficient quantity. Today, this method has a very important point in many aspects of genetic engineering, molecular biology, diagnostic microbiology, cancer and genetic diseases diagnosis, identification, criminology, sequencing, archeology, evolutionary study of organisms, etc. [22, 23].

Ermie and colleagues (2007) identified fungi producing aflatoxin by PCR. This study was carried out by primers for genes encoding enzymes in the aflatoxin biosynthesis pathway, including ver-1, nor-1, omt-1, and aflR gene, which has regulatory role for PCR. The results were compared with thin layer chromatography results as a common method for diagnosis. The DNA of 14 *Aspergillus flavus* isolates were extracted by phenol-chloroform method and tested with each pair of primers. The results showed that it is a rapid method for identifying aflatoxin producing strains of *Aspergillus flavus*, but it cannot completely identify them from the non- aflatoxin producing isolates, and further studies should be done to develop a suitable screening method [24]. In 2015, Karimi et al. studied aflatoxin in 48 samples of raw pistachio in Karimi Farm in Torbat Heydarieh with ELISA technique. The results showed that the minimum, maximum and average aflatoxin standard was 0.27.0.3.0.62 ± 0.95 ng / g in raw pistachios. The aflatoxin concentration of the tested samples was reported less than the national standard of 15 ng / g and the European standard of 10 ng / g [25]. In 2016, nida a shihabhamed et al. using a special primer for PCR method for the detection of *Aspergillus flavus*, studied aflatoxin production by determining isolates and identifying fungi from pistachio in local Babol markets in Iraq. The results of this study showed that *Aspergillus flavus* was identified in all types of nuts. The highest amount of this fungus was in pistachio with 70% and in the the rest of the nuts it was from 10 to 60%. In this study, PCR technique was used to detect aflatoxin from *Aspergillus flavus* and chemical ammonia detection was used for comparison with this technique [26]. In 2016, Adibian used several techniques such as thin layer chromatography (Tlc), HPLC, and ELISA to detect aflatoxin in pistachios. But these techniques were expensive and time consuming, while visual methods such as fluorescence spectroscopy by laser used in the HPLC system to increase the sensitivity of aflatoxin measurement are more rapid [27]. In this study, aflatoxin producing genes in *Aspergillus flavus*, in fresh pistachios in Kerman were studied.

Materials and Methods

In this descriptive cross-sectional study, 36 samples were collected from pistachios newly introduced into the market of Kerman. The total pistachios including green shell, firm shell and pistachio cereal in sterile containers and at 4 ° C were transferred to the laboratory. In the laboratory, the samples were transferred to the culture medium of Sabouraud dextrose broth and then the growth of bacteria was stopped by addition of chloramphenicol to this medium. They were incubated for 48 hours at a 30-28 °C, after which some of it was placed on the Rose Bengal agar and was placed in the incubator under heat, humidity and darkness for 7 to 10 days until the emergence of aspergillus colonies. In this step, homogenization was performed. The positive control used in this study was aflatoxin producing aspergillus strains and the negative control was non- aflatoxic strains of aspergillus. All of these 14 standard strains were obtained from Tehran University of Medical Sciences. After sedimentation, DNA extraction was performed on sediment. The 5 kits of respectively white, red, brown, blue and black cap were used from Cinnagen Company. A 250 µl of white cap (DM1) buffer was poured onto the precipitate and pepitted for 15 s, then 250 µl of the red cap (DM2) buffer was added to these solutions and pipetted for 30 s, then the micro-tube was centrifuged in 12000 rpm for 5 min and 400 µl of the supernatant was transferred into a new micro-tube. 400 µl of Brown cap (DM3) buffer was added to the supernatant, and then the solution was poured into a DNA spin column and centrifuged at 10000 rpm for 1 min at room temperature. 500 µl wash buffer (Blue cap) was added to the DNA spin column to wash and remove the ethanol in the sample and centrifuged for 1 min at 10000 rpm. Filter containing the DNA was placed in a new micro-tube and p30 µl of the Elution buffer (Black cap) was poured on the filter and was centrifuged after 3 min at 10000 rpm for 1 min and again 30 µl of elution buffer was poured vertically onto the filter and centrifuged after 3 min. DNA concentration was determined using a nanodrop spectrophotometer. DNA concentration of the positive control in this study was 56.5 ng / Ml at 260 nm.

The aflR gene, which is a synthesis pathway regulatory genes, was selected. The nucleotide sequence is as follows:

F: 5′-TATCTCCCCCCGGGCATCTCCCGG-3′

R: 5′-CCGTCAGACAGCCACTGGACACGG-3

The DNA content of the positive control sample was selected based on its concentration of 2 µL. In this study, dNTP solution with the concentration of 10 mM (Roche Germany) was used. In PCR assay performed, the concentration of 200 µm was required. The PCR test was performed with 1 unit (U1). To perform PCR, 2 µl of DNA and 17.5 µl of distilled water were added to micro-tubes (on ice), and in another micro-tube rack, 97.5 µl of PCR buffer, 25.29 µl of magnesium chloride, 19.5 µl of DNTPs, 24.3 µL of the F primer, 24.3 µL of the R primer, 19.5 µl of the taq polymerase enzyme (Roche Germany) were added. Then 5.5 µl of this micro-tube was added to each of the pre-prepared DNA and water samples, each of which was vortexed and spinned. The samples were taken from the ice rack and placed in the thermocycler. The time schedule and temperature for aspergillus was set and the device was turned on for one and a half to two hours. To prepare TAE buffer (25x), 5.60 g tris-base, 4.65 g EDTA, add 14.3 cc acetic acid were poured into a volumetric and reached to 500 ml volume with distilled water. It was placed on a magnetic stirrer to become clear. To prepare the TAE buffer (1x), 40 ml of TAE buffer (25x) was mixed with 960 ml of distilled water. The percentage of agarose gel in this study is one percent that agarose powder was weighed and the TAE buffer was added to it. One g of agarose powder was weighed and dissolved in 100 cc of buffer, then placed in a microwave to slightly boil and a smooth and uniform solution was obtained. Then, the suitable casting tray and well combs were selected in accordance with the sample and covered the casting tray with a tape to avoid from removing the agarose before getting solidified. Put the casting tray on a balanced place and adjust the well comb in it to become ready for pouring the gel. When the solution is slightly cooled, gently was poured into the castle tray to prevent the bubble forming and after 20 minutes the gel is completely solidified. After the gel is prepared, the tape was removed from the castle tray and the tank was filled with a TAE buffer. Then the well comb was removed slowly and wells were made in the gel. Diameter of gel is up to 5 mm. To transfer the PCR products to the wells in the gel, first, the DNA ladder (marker) from 3.5 to 3.7 µl, the cyber green color and the loading buffer were mixed on the aluminum paper with a pipettor , then the pipettor was kept vertically and slowly inserted into the well, the second sample, which was positive control, the asparagillus sample producing the aflatoxin was mixed with dye and loading buffer and inserted into the second well, and the third sample, which was negative control, was mixed with the dye and loading buffer and transferred to the well, then the unknown specimens in the same way were inserted into the wells. For this purpose bp100 marker was used. In the following, the tray castle was placed in a way in the electrophoresis tank so that the wells are placed on the side of the negative pole of the tank.
The tanks were connected to the current and the voltage was set to 100, and the amperage was approximately half of that. After 45 - 60 min, gel watch was checked and the colure that has progressed in the gel, the gel observation time could be estimated. After the electrophoresis, was followed by the gel was removed from the buffer and washed with water and was placed in a transilluminator UVP and after the observation on the monitor and adjusting the light, was pictured by a especial camera and printed.

Results

Of the 36 pistachio samples that by morphology, blackness and spots on their shelf were suspicious to fungal contamination, after culturing, observing aspergillus colonies (white and prominent, cloudy in rosebengal medium) and observing microscopic characterizes, 11 cases infected to different types of fungi such as aspergillus, mucur, penicillium and saprophytic fungi (30.55%) and 25 (69.45%) were non-contaminated. After that, hags were taken from the colonies grown in the first medium (Fig. 1) and in were cultured the second medium linearly to complete the isolation of colonies.

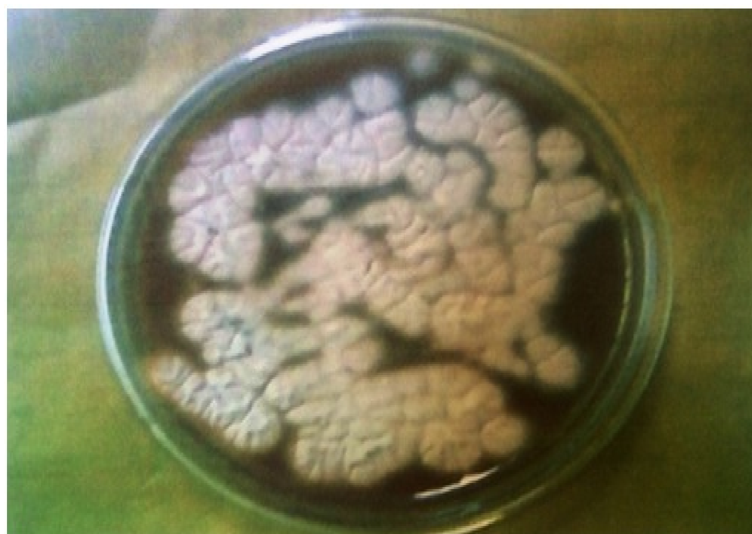

Figure 1: Bulk colonies of aspergillus in the mentioned medium

In the first culture, which was carried out on Rosebengal According to the results, the amount of fungi from contaminated samples was given as percentage in the following graph (Fig. 1).

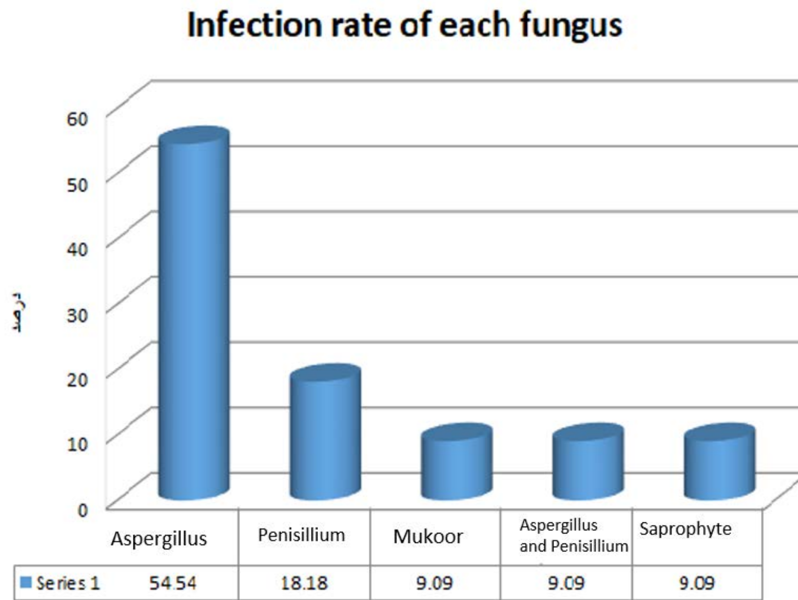

Figure 1: Percentage of infection of each fungus in infected samples

As shown in Fig. 1, in 11 infected samples, in 6 samples aspergillus (54.54%), in 2 cases penicillium (18.18%), in 1 case saprophyte (9.09%), in 1 case mucor (9.09%) and in 1 case penicillium mixed with aspergillus (9.09) were obtained. The molecular results of this study showed that from 7 samples of aspergillus, 4 samples had aflatoxin producing gene and 3 samples lacked the genes. Among all 36 samples tested, 11.1% of the samples had aflatoxin producing genes and among all samples contaminated with aspergillus, only 57.1% had aflatoxin producing genes. Also, the resultsof this study indicated that in 89 / 88% of the samples, aflatoxin producing genes were not present samples, aflatoxin and from all samples infected with aspergillus, 9.42 percent didn't have aflatoxin producing genes (42.9%) (Table 1).

Table 1: PCR results aflR target gene in all samples contaminated with different species of aspergillus

Microscopic observation	Aspergillus perentage	Total percantage	Sample number	
aspergillus	57.1	11.1	11-10-3-1	Presence of aflR gene
aspergillus	42.9	88.89	30-22-8	Lacke of aflR gene

In Fig. 2, Gel electrophoresis image of the PCR product is shown using the aflR primer.

Fig. 2: Gel electrophoresis image of the PCR product using the aflR primer (1032)

Discussion and conclusion

Pistachios can be exposed to various types of fungal infection, including aspergillus, penicillium, mucor, fusarium and yeasts with lower abundance [28]. Aspergillus is an ascomycete. Aspergillus has a mycelium structure with an intermediate blade, conidiaphore, vesicles, striagma and conidia, strigma in some species is one row and, in some species, two rows. It is a saprophytic and opportunistic fungi that infects the environment, food, vegetables, fruits, open cap drugs, dialysis bags, and air conditioning devices. Asparagillus has fluffy colonies that appear to be grainy and green to yellow [4, 6]. In the pathway for aflatoxin biosynthesis, housekeeping genes such as nor1, nor2, ver1, ver2, omtA ... and regulatory genes such as aflR are involved. aflR is a regulatory gene in the AF / ST biosynthesis pathway that is called AFL-2 in Aspergillus flavus. The role of this gene is activating the transcription in Aspergillus flavus and Aspergillus parasiticus. Another gene that is somehow involved in the regulation of transcription is aflJ. The genes involved in the synthesis pathway have roles from the conversion of the fatty acids at the beginning of the pathway to the production of aflatoxin as the final product of the pathway. In the absence of regulatory genes or their shutdown, the transcription does not occur, and despite the existence of all pathway genes, due to the disturbance in these genes, especially the aflR, the fungus is not capable of producing aflatoxin [29].

In general, because the aflR gene is a vital and important gene for the production of aflatoxin, the presence of this gene in the polymerase chain reaction ensure the presence of aflatoxin gene in our product. One of the toxic solutions that was used to extract DNA in the past was phenol chloroform, which was not used in this test because it was very toxic and time-consuming, as well as has a high percentage of error. Instead, a series of extraction kits with different colors were used according to the instructions. These buffers (including alcohol, lysis buffer, washing buffer, and elution buffer) had a much lower percentage of toxicity, higher precision and sensitivity and, more importantly, greatly increased the rapidity of DNA extraction (within half an hour).

In this study, Cyber Green was used to stain compared with Ethidium bromid, which has a high toxicity. The highest rate of infection in pistachio was by aspergillus, which suggests that pistachios or nuts are more susceptible to this fungus, which probably structural properties and sensitivity of this fungus to moisture and temperature, as well as the suitability of the fungus growth rate on pistachio more than other fungi are the cause of this infection increase. In this study, the infection of fresh pistachios in Kerman province, which is the main source of this product in the world, as well as pistachio, which is one of the most important dried fruits of the world known as green gold either in the food industry or in the cosmetics industry and exportation which is the largest non-oil exports in the world were studied considering fungal cinfetion and the presence of aflatoxin producing gene. The other results indicated that among 36 cases that were selected, 6 cases of aspergillus, 2 cases of penicillium, 1 mucurium, 1 case of penicillium and aspergillus, 1 case of Saprophytic fungus were observed, and only 4 cases of all samples and among the fungi, aspergillus was positive in case of Molecular assay and in 25 cases no fungal cinfection was observed and the samples were healthy. Therefore, deciding from the appearance of samples is not the only accurate diagnosis of fungal infection and it requires molecular and culture methods.

Four positive cases of aflatoxin producing gene were found in in Sirjan Mahan, Rafsanjan, and Kerman, where atmospheric conditions or treatment of farmers, the regional moisture content of these divisions compared to other parts such as Kahnouj, Rabat, Shahrokhabad, Chatrud, Zangi Abad and qudrat Abade Zarand, which are considered as a small divisions of the province, did not show any fungal infection.

The only primer used in this study to detect aflatoxin producing gene was the aflR regulatory gene for proving the presence of aflatoxin. Nasimpour et al., in Khorasan region of Gonabad and Feyzebad, identified 30 isolates of aspergillus genus and isolated the strains using methods based on culture and potato dextrose agar medium. The identification of the genus aspergillus was performed by using the polymerase chain reaction using the ASP1 / ASP2 primer pair for amplification of the 18s rRNA region. Also, genes involved in the production of aflatoxin were detected by 3 pairs of primers APA-450 / APA-1482 omt-208 / omt1232, ver1 / ver2. The results showed that among 30 isolates of fungi, 12 samples contained omtA gene and 4 samples contained ver1 gene. In none of the fungal agents of the regulation gene of aflR was observed.

The results showed that although some of isolates have two structural genes involved in aflatoxin biosynthesis pathway, due to the absence of aflR regulatory gene, they are able to produce aflatoxin. To investigate the possible relationship between the presence of aspergillus spp. and the aflatoxin producing genes in the pistachio, the correlation coefficient was calculated. The results of statistical analyzes showed that there is a high correlation between the presence of aspergillus spp. and omtA and ver1 genes based on the moisture content ($p < 0.05$). However, there is no significant relationship between the presence of aspergillus spp. and the mentioned genes in mold and yeast count and the total count of mesophile microorganisms [29].

Momen et al., reported the most common fungi isolated from pistachio samples in almonds and eggs, respectively, as aspergillus with 84.69%, penicillium with 9.3%, and mucor with 48.4%, which aspergillus is the most common genus which was consistent with this study, [30]. In a study by Rahimi et al., 11 species of aspergillus were identified and 3 genes of aflr, aflJ and omtB were used to determine the production of aflatoxin from isolates in PCR. In all of the studied samples, a primer was designed to amplify the omtB gene, which could proliferate a 611 bp fragment in the aflatoxigenic isolates in this study. According to the results of thin layer chromatography and fluorescence analyzes using beta-cyclodextrin in the culture media, using this method the presence of aflatoxin in pistachios produced in different regions of Iran was confirmed.

This result showed that the PCR method in combination with fluorescence is a simple and reliable confirmation test to show infection of pistachio with aflatoxin. Also, in a study conducted by Rahimi et al., the highest infection rate in the fresh

pistachios was with aspergillus with 74% which was in consistent with the findings of the current research. Also, the unit of DNA fragment was replicated by the aflR primer pair in *Aspergillus parasiticus* and 33 isolates from 100 isolates of *Aspergillus flavus*, which the only use of this primer in this section is also in agreement with our research [31]. Panjeke and colleagues isolated 22 aspergillus from the pistachios infected with fungi. Also especial primers were designed for the aflR gene, and the 798 bp band of each isolate in the amplification of DNA is the reason of the presence of an aflatoxin regulatory gene in the genome. Therefore, only aflR regulatory gene was used to detect aflatoxin producing aspergillus, which is in agreement with the present study [32].

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