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Molecular detection of mycobiota and aflatoxins contamination of chili

Molekularer Nachweis von Schimmelpilzen und Aflatoxinen in Chili

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Summary

Capsicum annuum (bell and chili peppers) is one of those vegetable crops growing in the warm areas. Pepper production conditions require drying fruits in most cases by the sun light. During drying processes, this crop is exposed to contamination with microorganisms, especially fungi. In this article, mycobiota of 60 samples of different chill products from retail markets and from food restaurants of Taif city was studied. Crushed chili showed high fungal load compared with chili sauce and chili powder, while chili powder showed high occurrence of aflatoxins. *Aspergillus, Eurotium* and *Penicillium* were the most common genera isolated from chili samples. The total aflatoxins potential of 35 isolates of *A. flavus, A. parasiticus* and *A. tamarii* were studied. Seventy percent of *A. flavus* isolates were aflatoxigenic. The frequencies of aflatoxin biosynthesis genes named *aflR, nor-1, ver-1* and *omtA* were studied in the aflatoxigenic species of *Aspergillus* collected in this study. Forty-six percent of aflatoxigenic *A. flavus* isolates and one non aflatoxigenic one showed DNA fragments that correspond to the complete set of genes. In conclusion, the high co-occurrence of *Aspergillus* species able to produce aflatoxins, particularly in chili samples, suggested the need of a more efficient control during processing and storage to reduce fungal contamination, and additional legislation to consider the simultaneous presence of aflatoxins in these matrices.

Keywords: *Aspergillus flavus,* Aflatoxin genes, Flourometer, co-occurrence, molecular markers

Zusammenfassung

Capsicum annuum (Spanischer Pfeffer, Paprika) gehört zu den Gemüsesorten, die in warmen Gegenden wachsen. Für die Chiliproduktion müssen die Früchte getrocknet werden, dies geschieht überwiegend mit Sonnenlicht. Während des Trockenvorgangs wird dieses Gemüse häufig von Mikroorganismen kontaminiert, in der Regel von Schimmelpilzen. In dieser Studie wurde das Schimmelpilzvorkommen von 60 Proben verschiedener, gekühlter Produkte von Kleinmärkten und Restaurants der Stadt Taif untersucht. Chiliflocken hatten eine höhere Pilzbelastung im Vergleich zu Chilisoße oder Chilipulver gezeigt. Während in Chilipulver hohe Aflatoxinwerte festgestellt wurden. *Aspergillus, Eurotium* und *Penicillium* waren in dieser Studie die am häufigsten isolierten Gattungen. Aflatoxin wurde in insgesamt 35 Isolaten von *A. flavus, A. parasiticus* und *A. tamari* festgestellt. Siebzig Prozent der isolierten *A. flavus*-Stämme bildeten Aflatoxine. Überwiegend wurden die Aflatoxin-Gene *aflR, nor-1, ver-1* und *omtA* in dieser Studie nachgewiesen. Abschließend lässt sich sagen, dass das gemeinsame Auftreten der *Aspergillus* Arten, die in der Lage sind Aflatoxine zu bilden, eine effizientere Kontrolle während der Verarbeitung und Lagerung notwendig machen.

Schlüsselwörter: *Aspergillus flavus,* Aflatoxingene, Fluorometer, molekulare Marker

Introduction

The pepper *(Capsicum annuum)* is one of those vegetable crops grown in the warm climates (Sreedhara et al., 2013). It constitutes about 34 % of the spice trade in the world, and the increase in the demand and consumption of the pepper is more than 5.2 % annually. The world production of chili crops sums up to around 7 million tones, which is cultivated on approximately 1.5 million hectares of land. From 2000 on, 10.35 thousand tons have been imported in Saudi Arabia. India is the largest producer of chili. Its production level ranges around 1.1 million tons annually. India also has the maximum area dedicated to the production of this crop. Chili is an important commodity used as a vegetable, spice, medicinal herb, and ornamental plant by billions of people every day (Rajeev, 2010). It is also used as an ingredient in industrial products. Both dry and green chilies are produced all over the world.

Spoilage caused by fungi during plant growth and the storage of agricultural products is should be avoided (Freire et al., 2000; Elshafie et al., 2002; Hashem and Alamri, 2010). Not only is the quality of the products decreased but also there is a risk to human and animal health from mycotoxins (Erdogan, 2004; Venâncio and Paterson, 2006; Salem and Ahmad, 2010; Zain, 2011).

Aflatoxins (AF) are human liver carcinogens (Liu and Wu, 2010). The mycotoxins are a major health threat for humans health because they are toxigenic, carcinogenic, mutagenic and teratogenic (Paterson and Lima, 2010). Aflatoxins commonly found are aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), and G2 (AFG2). AFB1 is the most potent of all aflatoxins known to date and is generally found in the highest concentration in food and animal feeds (Lee et al., 2004). These toxins are highly carcinogenic and elicit a wide spectrum of toxic effects when foods and feeds contaminated with aflatoxins were ingested (Peskta and Bonday, 1990).

Chilies have been reported as one of the crops most heavily contaminated with aflatoxins (Marin et al., 2009). Contamination of chili with aflatoxins and ochratoxins may take place in the field (pre-harvest) or during drying, storage or processing stages (post-harvest). Aflatoxins contamination was found to be higher in summer chili samples and hence, winter chilies may provide a better quality product with respect to aflatoxins contamination. Because of the toxic and carcinogenic potential of mycotoxins, there is an urgent need to develop detection methods that are rapid and highly specific (Konietzny and Greiner, 2003). Because mycotoxins have diverse chemical structures, it is not possible to develop one method to detect all relevant mycotoxins. The highly advanced physico-chemical methods for the analysis of mycotoxins in use have above all the disadvantage that highly sophisticated clean-up and/or derivatization procedures must be applied (Smith et al., 1994). At present, monitoring of agricultural commodities, foods and animal feeds for the presence of a fungal contamination includes cultivation and taxonomic identification at the morphological level (Liewen and Bullerman, 1999). This approach is, however, very time-consuming, labor-intensive, requires the expertise of mycologists, and, above all, possesses the inherent possibility of misclassification, since morphological characters of fungi can be highly variable depending on the media and culture conditions. Therefore, more rapid and more objective methods for the identification of mycotoxigenic fungi in human foods and animal feeds are needed for evaluating the microbiological risks of a given product (Konietzny and Greiner, 2003). Also, the most important aflatoxin producers from a public health point of view are members of *Aspergillus* section Flavi, in particular *A. flavus* and *A. parasiticus.* Originally, several isolates of *A. parasiticus* were misidentified as *A. flavus* (for example NRRL 2999, 3000 and 3145; Hesseltine et al., 1966; Applegate and Chipley, 1973).

Molecular techniques have been introduced as powerful tools for detecting and identifying fungi (Geisen, 1998). For this reason, rapid methods, including polymerase chain reactions (PCR), have been developed for direct testing of food for the presence of pathogens, as well as for confirmation and genotyping of isolates from foods. PCR-based assays for the detection of common foodborne pathogens are available from several manufacturers. The main advantage of PCR is that organisms need not be cultured, at least not for a long period prior to their detection. PCR-based methods that target DNA are considered a good alternative for rapid diagnosis because of their high specificity and sensitivity (Accensi et al., 1999; Rath and Ansorg, 2000; Schmidt et al., 2003; Perrone et al., 2004), especially when multi-copy sequences are used to develop species-specific primers (Bluhm et al., 2002).

PCR-based methods are considered a good alternative for rapid diagnosis of fungi because of their high specificity and sensitivity (Schmidt et al., 2003), especially when multi-copy sequences are used to develop species-specific primers (Bluhm et al., 2002). The biosynthetic pathway for aflatoxin production by *A. flavus* has been deciphered, and genes in the aflatoxin biosynthetic pathway have been identified (Payne et al., 1993; Trail et al., 1995; Yu et al., 1995, 2004). The AflR gene plays an important role in the aflatoxin biosynthetic pathway by regulating the activity of other structural genes such as *omt-A, ver-1* and *nor-1* (Woloshuk et al., 1994; Chang et al., 1999).

This study aims to: (1) determine the mycobiota of chili offered for sale to consumers in the retail stores at Taif region in Saudi Arabia, paying a special attention to the mycotoxigenic moulds; (2) evaluate the contamination levels of Aflatoxins in Chili samples; and (3) evaluate the presence and the frequencies of the PCR products corresponding to amplification of *aflR, nor-1, ver-1* and *omtA* genes in *Aspergillus Flavi* section isolated from chili samples.

Materials and Methods

Sampling

A total of 60 chili samples including chili sauce (n = 20), crushed chili (n = 20) and chili powder (n = 20) were purchased randomly from retail markets and from food restaurants of Taif city. The main ingredients of chili sauce were tomato paste, water, white vinegar, minced onion flakes, sweet chili powder, plain salt, garlic powder and red chili flakes. Samples comprised 500 g (cruhed chili and chili powder) and 200 mL (chili sauce). The samples were saved in plastic bags and stored in freezer at 4 °C until further analysis.

Mycobiota determination

Total fungal counts were performed on dichloran 18 % glycerol agar (DG18; glucose 10 g; peptone 5 g; KH_2PO_4 1 g; $MgSO_47H_2O$ 0.5 g; glycerol 220 g; agar 15 g, dichloran

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2 mg; chloramphenicol 100 mg; and H_2O 1 L), a medium that has lower water activity (a_w = 0.955) and favours xero philic fungi development (Pitt and Hocking, 1997). Quantitative enumeration was done using the surface-spread method. Ten grams of each sample were homogenized in 90 mL of peptone water solution. Serial dilutions were made, and a 0.1 mL aliquot was inoculated in duplicate onto the culture media. All the plates were incubated in the dark at 25 °C for 5–7 days. Only plates containing 15–150 colony-forming units (CFU) were used for counting and the results were expressed as CFU per gram of sample. All colonies were transferred for sub-culturing to plates of MEA. Taxonomic identification of the different genera and species was made according to macroscopic and microscopic criteria with appropriate keys (Nelson et al., 1983; Pitt and Hocking, 1997; Samson et al., 2000; Klich, 2002). After taxonomic identification, all *Aspergillus* section *Flavi* were subjected to confirmation by molecular analysis with specific primers for these species described in previous studies (González-Salgado et al., 2008).

Detection of natural occurrence of total aflatoxins in chili samples

The samples were analyzed for total aflatoxins using a slightly modified immunoaffinity method based on Association of Official Analytic Chemists (AOAC) method (Trucksess et al., 1991). As previously described by Lewis et al. (2005), the whole sample was ground and a 100 g subsample was removed for analysis. Methanol: water (80:20) solvent (100 ml) and 5 g NaCl were added to each sample and the mixture was blended at high speed for 3 min. The mixture was then filtered through a fluted filter paper (Whatman 2V, Whatmanplc, Middlesex, UK), and the filtrate was diluted (1:4) with water and refiltered through a glass-fiber filter paper. Two milliliters of the glass-fiber filtrate were placed on AflaTest®WB SR Column (VICAM, Watertown, MA, USA) and allowed to elute at 1–2 drops/s. The columns were washed two times with 5 ml water, and aflatoxin was eluted from the column with 1 ml high performance liquid chromatography (HPLC)-grade methanol. A bromine developer (1 ml) was added to the methanol extract, and the total aflatoxin concentration was read in a recalibrated VICAMSeries-4 fluorometer set at 360 nm excitation and 450 nm emissions.

Determination of total aflatoxinogenic abilities of *Aspergillus flavus, A. parasiticus* and *A. tamarii* isolates

The test for the aflatoxin-producing ability of the isolates was performed by cultivating the fungal strains in Czapek Yeast extract agar (Ben Fredj et al., 2009) medium for 5 days at 25 ± 2 °C. Three replications were maintained for each isolate for each media. Total AFs were extracted by grinding the moldy agar (20 g) in waring blender for 5 min with methanol (100 ml) containing 0.5 % NaCl, and the above-mentioned procedure was applied.

Molecular detection of mycotoxin-producing genes

The isolation of DNA from mycelia was performed according to the method described by Farber et al. (1997). Four published primer sets were used for the specific detection of *nor-1, ver-1, omt-A* and *aflR* genes (Criseo et al., 2008). The 400, 537, 797 and 1032 bp fragments were amplified, respectively. A typical PCR was carried out under the following condition: 5 µl of genomic DNA were used as template (2 µg ml^{-1}), 0.5 U EuroTaq polymerase (Euroclone, Pero-Milan, Italy), 1 x reaction buffer, 2.5 mM $MgCl_2$, 200 µM each dNTP and 7.5 pmol each primer in a total reaction volume of 50 µl. A total of 35 PCR cycles with the following temperature regimen was performed: 95 °C, 1 min; 65 °C, 30 s; 72 °C, 30 s for the first cycle; and 94 °C, 30 s; 65 °C, 30 s; 72 °C, 30 s for the 34 left (Criseo et al., 2008). PCR products were separated on a 1.3 % (wt/vol) agarose gel and stained with ethidium bromide.

Results and Discussion

Mycobiota of different chili samples

In general, crushed chili samples were more contaminated than other samples (mean total 11.335 x 10^3 CFU/g crushed chili) as shown in table 1). Santos et al. (2011) reported that smoked paprika samples were more contaminated with fungi than the other samples (1.9 x 10^3 CFU/g in DG18), i. e. paprika (3.8 x 10^2 CFU/g) and chili samples (1.3 x 10^2 CFU/g of chili). The lower fungal counts in chili sauce (0.049 x 10^3) could be attributed to presence of vinegar in the ingredients of this product of the chili.

TABLE 1: *Mean values of total fungal counts, occurrence of Aspergillus spp.on three different chili products and incidence range of total aflatoxins (AFs) in chili products.*

Sample types	Total fungal counts [CFU/g] Mean value ± Standard deviation	*Aspergillus* spp. occurrence [%]	Positive samples*	Range of AFs [ppb]	Mean of AFs [ppb] ± Standard deviation
chili sauce	49 ± 11.0	5	0	0	0
crushed chili	11335 ± 65.0	35	16	20–170	15 ± 0.9
chili powder	9771 ± 42.0	27	18	35–200	17 ± 2.1
Total	**21155 ± 74**	**67**	**34**	**0–200**	**16 ± 3.0**

* Out of 20 samples from each type of chili product.

Eleven genera comprising 26 species of fungi were recovered from 60 samples of chili collected from retail markets and food restaurants of Taif city during 2013 (Table 2). The predominant mycobiota of chili samples, taking into account their occurrence and abundance, respectively, *Aspergillus* spp. (65.49 %, 70 %), *Eurotium* spp. (9.92 %, 40 %), *Penicillium* spp. (7.80 %, 30 %), *Mucor* sp. (3.78 %, 15.00 %), *Alternaria* sp. (3.55 %, 16.67 %), *Mycosphaerella* sp. (3.31 %, 13.33 %) and the remaining genera were isolated in low or moderate frequency comprised collectively 6.15 %. All *Eurotium* species isolated in this study are now listed under *Aspergillus* according to the Index Fungorum. So, the collective results indicated that *Aspergillus* was the most frequently isolated genus in this study. The occurrence and abundance of this genus were 75.4 % and 86.7 %, respectively. The same results were obtained by Hashem and Alamri (2010), during their studies in mycobiota of chili in Aseer region (Saudi Arabia), having isolated *Aspergillus awamori, A. flavus, A. niger, A. ochraceus, A. amarii, Eurotium re-*

TABLE 2: *Occurrence, average total counts (calculated per g sample), abundance, number of cases of isolation and frequency of collected species from 60 of different chili samples.*

Fungal genera & species	Chili products*	ATC	Abundance	NCI	F%
Alernaria alternata	C+P	750	3.55	10	16.67
Aspergillus	S+C+P	13855	65.49	42	70
A. candidus	S+C+P	150	0.71	5	8.33
A. carbonarius	C+P	600	2.84	8	13.33
A. flavus	S+C+P	11500	54.36	30	50
A. niger	S+C+P	850	4.02	13	21.67
A. parasiticus	C+P	150	0.71	2	3.33
A. niveus	C	5	0.02	1	1.67
A. ochraceus	S+C	150	0.71	2	3.33
A. tamarii	C+P	250	1.18	3	5
A. terreus	C	50	0.24	1	1.67
A. ustus	C	150	0.71	2	3.33
Botrytis cinirea	C+P	100	0.47	2	3.33
Eurotium	S+C+P	2100	9.92	24	40
E. amstelodami	S+C+P	900	4.25	12	20
E. chevalieri	S+C+P	500	2.36	6	10
E. repens	C+P	400	1.88	3	5
E. rubrum	C+P	300	1.42	5	8.33
Fusarium oxysporum	S+C+P	550	2.60	7	11.67
Mucor racemosus	S+C+P	800	3.78	9	15
Mycosphaerella tassiana	C+P	700	3.31	8	13.33
Penicillium	S+C+P	1650	7.80	18	30
P. chrysogenum	S+C+P	1000	4.73	10	16.67
P. corylophilum	C+P	300	1.42	4	6.67
P. citrinum	C	150	0.71	2	3.33
P. griseofulvum	C+P	200	0.95	3	5
Rhizopus stolonifer	C+P	400	1.88	5	8.33
Scopulariopsis brevicaulis	C	200	0.95	2	3.33
Ulocladium chartarum	C	50	0.24	1	1.67
Total		**21155**	**100**		

*S = chili sauce, C = crushed chili and P = chili powder.

TABLE 3: *Co-occurrence of Aspergillus species in chili samples. Values in brackets are percentages of the total samples analyzed.*

Sample	No. species per sample (%)					
	0	1	2	3	4	5
chili sauce	15 (75)	2 (10)	3 (15)	0	0	0
crushed chili	1 (5)	3 (15)	4 (20)	5 (25)	6 (30)	1 (5)
chili powder	2 (10)	3 (15)	4 (20)	6 (30)	4 (20)	1 (5)
Total	**18 (30.00)**	**8 (13.33)**	**11 (18.33)**	**11 (18.33)**	**10 (16.66)**	**2 (3.33)**

annum and *C. frutescens.* Gomathi et al. (2011) studied monthly variation of fungal population in chili field at 4 different districts (Thiruvarur, Nannilam, Kudavasal and Valangaiman) in India. They isolated 40 different fungal species, and the dominant genera recorded were *Aspergillus* and *Penicillium.* Freire et al. (2000) isolated a wide range of field and storage fungi (42 species) from black and white pepper from Amazonia, with *Aspergillus flavus* and *A. niger* having been the most frequently isolated species.

Table 3 showed the co-occurrence of *Aspergillus* spp. in chili samples, where 30 % of the samples were not contaminated with *Aspergillus* spp., but 70 % of the samples contaminated with *Aspergillus* spp. Of those, 13.33 % of samples were contaminated by only one species, 18.33 % by two species, 18.33 % by three, 16.66 % by four, and 3.33 % by five different species. In Spain, Sardiñas et al. (2011) reported that the percentage of non-contaminated chili samples with aspergilla was 11 (35.5 %), while 9 (29.0 %) and 11 (35.5 %) samples were contaminated with one and two *Aspergillus* species, resp.

Among these *Aspergillus* spp., only three species (*A. flavus, A. parasiticus* and *A. tamari*) are well-known as aflatoxin producers. They were isolated from 30, 2 and 3 samples out of 60 samples of the different chili products, respectively (Tab. 4). Using PCR technique, Santos et al. (2011) proved that *A. flavus* DNA was detected in 82 % of chili, 60 % of paprika, and in all smoked paprika samples, while the *A. parasiticus* DNA was detected in only one chili sample.

Natural occurrence of total Aflatoxins in chili samples

Since the studied samples showed high contamination with *Aspergillus* spp. that eventually may be aflatoxinogenic, the contamination of samples with aflatoxins was also studied. 34 samples out of 60 resulted naturally contaminated with aflatoxins (Tab. 1). The percentages of contaminated samples were 80 and 90 % of crushed and powdered chili, respectively. The concentration of AFs in crushed chili samples ranged from 20–170 ppb, while this range was from 35 to 200 ppb from powdered chili samples. There was no aflatoxin contamination in sauce samples, while mean AFs levels in powdered and crushed chilies were 17 and 15 PPB, respectively (Tab. 1). European Union (EU) has set an acceptable level of AFs

pens, Paecilomyces lilacinus, Penicillium arenicola, P. corylophilum, P. dunkii, P. funiculosum, P. oxalicum, P. waksmani, Rhizopus stolonifer, Scopulariopsis brevicaulis, Trichoderma harzianum, and *Ulocladium botrytis* from pepper samples in PDA medium. Atanda et al. (1990) isolated 7 mould species from dry 'tatase' pepper and *Aspergillus niger, A. flavus* and *Geotrichum candidum* were the most prevalent species. Adegoke et al. (1996) isolated *Rhizopus oryzaze, Aspergillus niger, A. flavus, Geotrichum candidum* and *Saccharomyces* sp. as dominant fungi from *Capsicum*

TABLE 4: *The frequencies of occurrence of mycotoxigenic species detected in this study.*

Samples	Mycotoxigenic species		
	A. flavus	*A. parasiticus*	*A. tamarii*
chili sauce	1	0	0
crushed chili	14	1	1
chili powder	15	1	2
Total	**30**	**2**	**3**

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for spices at 5 µg kg^{-1} for AFB1 and 10 µg kg^{-1} for total AF (Commission Regulation, 2010) and our results indicated the contamination of chili samples were higher than this level. Hell et al. (2009) reported that chili samples were naturally contaminated with aflatoxin B1 and aflatoxin B2, at concentrations of 3.2 µg/kg in Benin, Mali and Togo. In Pakistan, Russell and Paterson (2007) studied aflatoxins contamination in chili samples from Pakistan. They reported that all investigated chili samples contained high levels of AFB1. Zinedine et al. (2006) analyzed spices' samples for aflatoxins and the average contaminations found for AFB1 were 0.09, 0.63, 2.88 and 0.03 µg/kg for black pepper, ginger, red paprika and cumin, respectively. The effect of chili season on contamination with aflatoxins was studied by Iqbal et al. (2011) in Pakistan. Their results reported that limits of detection and quantification for AFB1 and AFG1 were 0.05 µg/kg and 0.50 µg/kg, whilst for AFG2 and AFB2 they were 0.10 µg/kg and 0.60 µg/kg, resp. In the winter samples, AFs were detected in 18 (72 %) whole and 14 (60 %) ground chilies, with concentration ranges 0.00–52.30 µg/kg and 0.00–74.60 µg/kg, respectively. Recently, Khan et al. (2014) collected 331 red chili samples (226 whole, 69 powdered and 36 crushed) from all over Pakistan for the estimation of total aflatoxin contamination by thin layer chromatography (TLC). Their results indicated that mean AFs levels in whole, powdered and crushed chillies were 11.7, 27.8 and 31.2 µg/kg, respectively.

TABLE 5: *Frequency of single genes in Aspergillus flavus, A. parasiticus and A. tamarii isolates collected from chili samples.*

Strains code	Source of isolation	Total aflatoxins	Aflatoxin genes			
			aflR	*omt-A*	*ver-1*	*nor-1*
TUAf1	chili powder	+	+	+	+	+
TUAf2	crushed chili	+	+	+	+	+
TUAf3	chili powder	+	+	+	+	+
TUAf4	chili powder	+	+	+	+	+
TUAf5	chili powder	+	+	+	+	+
TUAf6	crushed chili	–	–	+	–	+
TUAf7	chili powder	+	+	+	+	+
TUAf8	crushed chili	–	+	–	–	–
TUAf9	crushed chili	+	+	+	+	+
TUAf10	chili powder	+	+	+	+	+
TUAf11	chili powder	+	+	+	+	+
TUAf12	chili powder	+	+	+	+	+
TUAf13	crushed chili	–	+	–	+	–
TUAf14	chili powder	+	+	+	+	+
TUAf15	chili sauce	–	+	+	+	–
TUAf16	crushed chili	+	+	+	+	+
TUAf17	chili powder	+	+	+	+	+
TUAf18	crushed chili	+	+	+	+	+
TUAf19	chili powder	+	+	+	+	+
TUAf20	crushed chili	–	–	+	–	+
TUAf21	crushed chili	–	+	+	–	–
TUAf22	chili powder	+	+	+	+	+
TUAf23	chili powder	+	+	+	+	+
TUAf24	crushed chili	+	+	+	+	+
TUAf25	crushed chili	–	–	+	–	–
TUAf26	crushed chili	+	+	+	+	+
TUAf27	crushed chili	–	–	–	–	+
TUAf28	chili powder	+	+	+	+	+
TUAf29	chili powder	+	+	+	+	+
TUAf30	crushed chili	–	+	+	+	+
TUAp1	crushed chili	+	+	+	+	+
TUAp2	chili powder	+	+	+	+	+
TUAt1	crushed chili	+	–	–	–	–
TUAt2	chili powder	+	–	–	–	–
TUAt3	chili powder	+	–	–	–	–
Total (35)		**26**	**28**	**29**	**26**	**27**

+ = PCR amplification signal present. – = PCR amplification signal absent.

Total aflatoxin potentials of *Aspergillus flavus, A. parasiticus* and *A. tamarii* isolates

A total 37 *Aspergillus* spp. isolates, i. e. *A. flavus* (30 isolates), *A. parasiticus* (2) and *A. tamarii* (5) were subjected for detecting their aflatoxigenic potential. They varied in their abilities to produce total aflatoxins (Tab. 5). Twenty-one isolates (70 %) of *Aspergillus flavus* isolated from powdered (15 isolates) and crushed (6 isolates) chili showed aflatoxin potentials. All *Aspergillus parasiticus* (2 isolates) and *A. tamarii* (3) were aflatoxigenic (Tab. 5). In the Sultanate of Oman, Elshafie et al. (2002) recorded that nine isolates (45 %) of the 20 *A. flavus* strains isolated from species screened were aflatoxigenic. Rajasinghe et al. (2009) reported that some *Aspergillus flavus* strains isolated from chili powder in Sri Lanka showed aflatoxinogenic potentials. Approximately, 68 % of *A. flavus* isolates from different pepper types in Nigeria produced aflatoxins in neutral red desiccated coconut agar (Ezekiel et al., 2013). Saadullah (2013) reported that 91 out of 113 isolates of *Aspergillus flavus* and all isolates of *A. parasitcus* (80 isolates) isolated from dried vine fruits in Iraq were aflatoxigenic, while none of *A. tamari* isolates (18) was. In Iraq,

Mohammed et al. (2010) showed 81.8 % of isolates of *A. flavus* isolated from different agricultural commodities had aflatoxigenic ability. In Saudi Arabia, Gashgari et al. (2010) reported that 7 isolates of 29 *A. flavus* strains isolated from wheat flour were aflatoxigenic. Also, seven out of 18 *A. flavus* isolates from date palm in Saudi Arabia had AFs potentials (Gherbawy et al., 2012).

PCR amplification of the aflatoxin structural genes

Finally, molecular techniques were used to confirm the abilities of the aflatoxigenic isolates of *Aspergillus* spp. in the genomic level.Twenty-five identified genes clustered within a 70-Kb DNA region in the chromosome are involved in the biosynthesis of AFB1, and their DNA sequences were already published (Yu et al., 2004, Criseo et al., 2001, Scherm et al., 2005). PCR was used for the detection of aflatoxigenic aspergilla based on the intermediated enzymes including norsolorinic acid reductase-encoding gene *nor-1,* the versicolorina dehydrogenase-encoding gene *ver-1,* the sterigmatocystin 0-methyl transfrase-encoding gene *omt-1* and the regulatory gene *aflR* (Erami et al., 2009).

Polymerase chain reaction (PCR) was applied using four sets of primers for different genes involved in the aflatoxin biosynthetic pathway. Bands of the fragments of *aflR, omt-1, ver-1* and *nor-1* genes can be visualized at 1032, 797, 537 and 400-bp, respectively (Fig. 1). All aflatoxigenic and non-aflatoxigenic isolates yielded different DNA banding patterns, with a number of bands ranging from zero to four (Tab. 5).

Twenty out of thirty aflatoxigenic *Aspergillus flavus* isolates (46.66 %) showed DNA fragments that correspond to the complete set of genes. Also, *Aspergillus parasiticus* isolates (2) showed the complete set of investigated genes (Tab. 5). The presence of four targeted genes confirmed the abilities of isolates to produce aflatoxins as previously mentioned by other researchers (Geisen, 1996; Criseo et al., 2001; Scherm et al., 2005; Rashid et al.,2008; Gherbawy et al., 2012).

Isolate TUAf30 isolated from crushed chili showed the complete set of investigated genes, although this isolates is non-aflatoxigenic. These results were in harmony with those of Criseo et al. (2001) whereas they detected some *A. flavus* strains that showed a complete set of genes but do not produce aflatoxins. Yang et al. (2004) reported different results by multiplex-PCR and enzyme-linked immunosorbent assay (ELISA). They stated that, although *norA, ver1, omtA* and *avfA (aflR)* genes had been detected in all tested strains, some of these samples were negative for aflatoxin detection.

The results of this work indicated clearly that the presence of four tested genes is no sufficient marker for the differentiation between aflatoxigenic and non-aflatoxigenic isolates. Other studies (Flaherty and Payne, 1997; Chang et al., 1999 a,b, 2000; Cary et al., 2002; Takahshi et al., 2002; Ehrlich et al., 2003) suggest that regulation of aflatoxin biosynthesis in *Aspergillus* spp. involves a complex pattern of positive and negative acting transcriptional regulatory factors, which are affected by environmental and nutritional parameters. Geisen (1996) suggests that the lack of aflatoxin production could also be due to simple mutations including substitution of some bases and Liu and Chu (1998) suggested that a variety of different physiological conditions affecting aflatoxin biosynthesis.

A second group constituted by one isolate (12.5 %) of non-aflatoxigenic *A. flavus* isolates showed three DNA banding patterns corresponding to *aflR, omt-A* and *ver-1* (Tab. 5), while a third group constituted by 4 (20 %) of non aflatoxigenic *A. flavus* isolates yielded two DNA banding patterns. *Omt-A* and *ver-1* were amplified from 2 isolates, TUAf13 contained *aflR* and *ver-1,* while TUAf21 showed *aflR* and *omt-1* genes. The last group containing 2 isolates showed single gene amplicon. TUAf8 and TUAf27 showed the presence of *aflR* and *nor-1* genes, respectively (Tab. 5). Criseo et al. (2001) reported that aflatoxin biosynthesis in *A. flavus* is strongly dependent on the activities of regulatory proteins and enzymes encoded by four genes named *aflR, nor-1, ver-1* and *omt-A*. By using specific PCR-based methods, they proved that the aflatoxigenic *A. flavus* isolates always show the complete gene set, whereas non-aflatoxigenic isolates lacking one, two, three or four PCR products indicating that the genes do not exist in these strains or that the primer binding sites changed.

Generally, *omt-A* was the most prevalent gene in tested isolates of this study. This gene was recovered from 27 isolates of *Aspergillus flavus* (out of 30) and 2 isolates of *A. parasiticus. Nor-1, aflR* and *ver-1* genes were recovered from 25, 26 resp. 24 isolates of aflatoxigenic and non-aflatoxigenic isolates of *A. flavus.* In Italy, Criseo et al. (2008) used 134 non-aflatoxin-producing strains of *A. flavus* isolated from food, feed and officinal plants to study the different genes involved in aflatoxin biosynthetic pathway. Their results indicated that the *nor-1* gene was the most representative (88 %) among the four aflatoxin structural assayed genes followed by *ver-1* and *omt-A* that were found with the same frequencies (70.1 %), while a lower incidence (61.9 %) was found for the *aflR* gene.

The PCR protocol used in this study failed to amplify any DNA pattern for the targeted genes in *Aspergillus tamarii* isolates (Fig. 1). On the contrary, under the high stringency conditions, *A. tamarii* DNA hybridized to all four of the *A. flavus* and *A. parasiticus* gene probes, indicating strong similarities in the biosynthetic pathway genes of these three species (Klich et al., 2000).

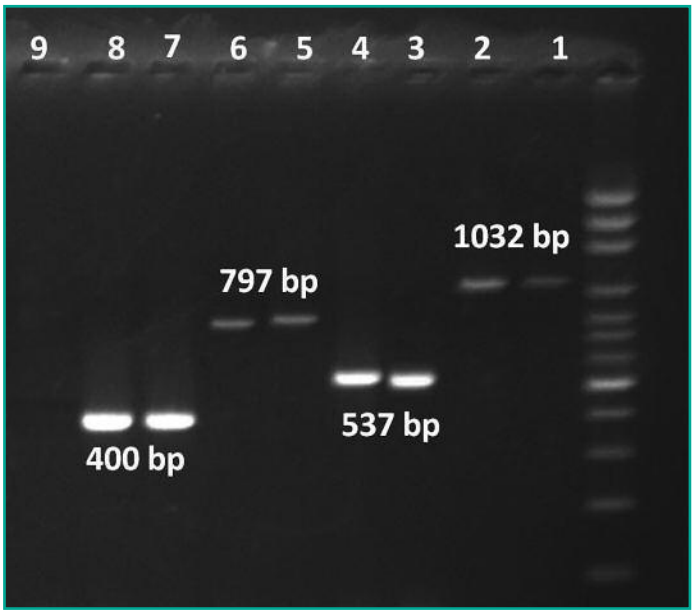

FIGURE 1: *Agarose gel electrophoresis of PCR products of four DNA fragments specific for aflR, nor-1, ver-1, and omt-A genes. Lane 1: Aspergillus flavus (aflR gene); Lane 2: Aspergillus parasiticus (aflR); Lane 3: A. flavus (ver-1); Lane 4: A. parasiticus (ver-1); Lane 5: A. flavus (omt-A); Lane 6: A. parasiticus (omt-A); Lane 7: A. flavus (nor-1); lane 8: A. parasiticus (nor-1), Lane 9: A. tamarii.*

In conclusion, the studied samples showed high contamination with aflatoxigenic species of *Aspergillus* spp. AFs levels in the investigated samples were higher than the acceptable levels. The PCR-based method used in this study confirmed the abilities of *Aspergillus* spp. to produce AFs. Due to the presence of aflatoxigenic aspergilla that caused contamination of chili products with aflatoxins in Saudi Arabia, further investigation is needed for monitoring and routine analysis. Furthermore, proper harvesting, drying, handling, storage and transport conditions need to be employed.

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Conflict of interest

The authors declare that no conflicts of interest exist.

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