PAPER

MOLECULAR CHARACTERIZATION OF AFLATOXIGENIC ASPERGILLI-CONTAMINATED POULTRY AND ANIMAL FEEDSTUFF SAMPLES FROM THE WESTERN REGION OF SAUDI ARABIA

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ABSTRACT

The aflatoxigenic abilities of 64 and 17 isolates of *Aspergillus flavus* and *A. parasiticus* isolated from poultry and animal feedstuff samples collected from the western region of Saudi Arabia were studied. Thirty-three (51.6%) and 13 (76.5%) isolates of *A. flavus* and *A. parasiticus*, respectively, were aflatoxigenic. The ranges of aflatoxins in *A. flavus* and *A. parasiticus* isolates were 4.4-110 and 143.6-271.3 ppm (µg/g), respectively. *A. parasiticus* isolates generally produced a greater amount of aflatoxins than *A. flavus*. *A. flavus* isolates from poultry, cattle, and camel and cattle feeds produced aflatoxin amounts in the range 5.7-110, 4.4-19.0, and 7.0-28.5 ppm, respectively. From poultry feedstuff samples, *A. parasiticus* produced aflatoxins in the range 212.5-232.4 ppm. Some aflatoxin biosynthesis genes (*aflR*, *omt-1*, *ver-1*, and *nor-1*) were detected with variable frequencies in all *A. flavus* and *A. parasiticus* isolates. The genetic diversity among 64 isolates of *A. flavus* using internal transcribed spacer sequence results and the amplification of some aflatoxin biosynthesis genes revealed that the investigated isolates showed high heterogeneity.

- Keywords: *Aspergillus flavus,* Aflatoxin genes, Flourometer, molecular markers, genetic diversity -

INTRODUCTION

Aflatoxin contamination of agricultural commodities has gained global significance as a result of their deleterious effects on human and animal health as well as their importance in international trade. The contamination of foods by aflatoxigenic fungi, particularly in tropical countries, may occur during pre-harvesting, processing, transportation, and storage (ELLIS *et al.*, 1991; MANONMANI *et al.*, 2005). Regular monitoring of toxigenic mycobiota in agricultural-based feeds and foods is an essential pre-requisite in the development of strategies to control or prevent mycotoxin exposure of feed animals and human population. Studies on the prevalence of toxigenic mycobiota of animal/poultry feeds have been regularly and frequently reported, including studies from Brazil (OLIVEIRA *et al.*, 2006; ROSA *et al.*, 2006), Argentina (DALCERO *et al.*, 1997), Nigeria (OSHO *et al.*, 2007), Spain (ACCENSI *et al.*, 2004), and Pakistan (SALEEMI *et al.*, 2010).

The polymerase chain reaction (PCR) first described by SAKI *et al.* (1985) requires the presence of specific target sequences. When genes involved in the biosynthetic pathway are known, they represent a valuable target for the specific detection of toxigenic fungi. The first researchers to use this approach for the detection of toxigenic fungi were GEISEN *et al.* (1996) and SHAPIRO *et al.* (1996), describing a diagnostic PCR directed against DNA sequences in the aflatoxin biosynthetic gene cluster. However, when the genes responsible for mycotoxin production are unknown, other sequences can function as a target. Examples are rDNA sequences, genes, or anonymous DNA marker sequences. GEISEN (1998) and EDWARDS *et al.* (2002) reviewed available diagnostic PCRs for toxigenic fungi. The advantages of the PCR-based approach for the detection of toxigenic fungi compared with those of the classical mycological or chemical analysis is mainly the time aspect. For the chemical analysis of mycotoxins in food, elaborated protocols for sample preparation and expensive laboratory equipment are necessary. Classical mycological analysis requires the isolation and cultivation of the fungi on different media and at least one week of growth for their reliable identification. In addition, much expertise is required to recognize the species, particularly for the main genera of toxigenic fungi *Fusarium*, *Penicillium*, and *Aspergillus*. In contrast, DNA extraction from food samples and raw materials of food can be performed in a few minutes (KNOLL *et al.*, 2002a). Further, the use of modern thermocyclers can reduce analysis time to less than 1 h (KNOLL *et al.*, 2002b).

The aflatoxin biosynthetic pathway involves approximately 25 genes clustered in a 70 kb DNA region (YU *et al.*, 2004). *A. flavus*, *A. parasiticus*, and other *Aspergillus* section *Flavi* species share nearly identical sequences and conserved gene order in the cluster. In recent years, PCR detection of aflatoxin biosynthetic gene presence or expression has been used as a diagnostic tool for aflatoxigenic fungi in selected food commodities (GEISEN, 2007; GALLO *et al.*, 2012).

Aflatoxins are regarded as potent hepatocarcinogens and immunosuppressants, and there are reports showing that this group of mycotoxins poses the biggest threat to the poultry and livestock industry through low productivity and death (VAN EGMOND, 1989; CHUKWUKA *et al.*, 2010; PEDROSA and BORUTOVA, 2011). Therefore, the potential risks of aflatoxicosis in Saudi poultry and livestock must be clearly evaluated in order to ensure prompt legislative action and mitigation of aflatoxin contamination in feed. This study was designed to determine and evaluate the aflatoxin-producing potentials of *Aspergillus* section *Flavi* isolated from poultry and animal feedstuff samples collected from the western region of Saudi Arabia. Furthermore, the isolates were tested for the presence of four of the characterized aflatoxin biosynthetic genes in their genome in relation to aflatoxin production.

MATERIALS AND METHODS

Samples

Sixty-four *A. flavus* and 17 *A. parasiticus* isolates were used throughout this investigation. These isolates were retrieved from poultry and animal feedstuff samples collected from the western region of Saudi Arabia (Taif, Makkah, and Jeddah). The isolates were identified according to their morphological features as well as sequence results of internal transcribed spacer (ITS) regions. The sequence results were deposited in the GenBank.

Determination of total aflatoxin abilities of *Aspergillus* species isolates

The aflatoxin-producing abilities of the isolates were determined by cultivating the fungal strains in Czapek Yeast extract agar (BEN FREDJ *et al.*, 2009) medium for 5 days at 25±2°C. Total aflatoxins were extracted by grinding the moldy agar (20 g) in a Waring blender for 5 min with methanol (100 mL) containing 0.5% NaCl. 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 re-filtered 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 twice with 5 mL of 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 (LEWIS *et al.*, 2005).

Molecular detection of aflatoxin biosynthetic genes in Aflatoxigenic species of aspergilli

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 conditions: 5 μL of genomic DNA were used as a template (2 μg/mL), 0.5 U EuroTaq polymerase (Euroclone, Pero-Milan, Italy), 1 × reaction buffer, 2.5 mM $MgCl_2$, 200 μM of each dNTP, and 7.5 pmol of each primer, in a total reaction volume of 50 μL. A total of 35 PCR cycles with the following temperature regimen were 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 remaining cycles (CRISEO *et al.*, 2008). PCR products were separated on a 1.3% (wt/vol) agarose gel stained with ethidium bromide.

Statistical analysis of frequency of aflatoxin biosynthetic genes

Cluster analysis of data was performed by hierarchical cluster analysis (SPSS Software, SPSS Inc., USA; Norusis, 1993).

RESULTS AND DISCUSSION

Total aflatoxin potentials of *Aspergillus* species isolates

Thirty-three out of 64 (51.6%) and 13 out of 17 (76.5%) of *A. flavus* and *A. parasiticus* isolates were aflatoxigenic producers, respectively. The aflatoxin range in *A. flavus* and *A. parasiticus* isolates was 4.4-110 and 143.6-271.3 ppm (μg/g), respectively (Tables 1 and 2). DUTTA and DAS (2001) carried out a groundwork study, in which 256 feed samples collected from different parts of Northern India were analyzed for aflatoxigenic strains of *A. flavus/parasiticus* and for detection of AFB1. Out of 198 *A. flavus* and 15 *A. parasiticus* strains isolated, 76% and 86%, respectively, were found to be toxigenic. RAZZAGHI-ABYANEH *et al.* (2006) surveyed the distribution of *Aspergillus* section *Flavi* in cornfield soils in Iran and their results indicated that only 27.5% of *A. flavus* isolates were aflatoxigenic (B1 or B2 or both), and all the *A. parasiticus* isolates produced aflatoxins of both B (B1 and B2) and G (G1 and G2) types. PITT (1993) also, reported that *A. flavus* isolates produced B1 and B2 or both types, while *A. parasiticus* produced the four aflatoxin types. These results support the present findings indicating that the level of aflatoxin production by *A. parasiticus* was higher than that by *A. flavus* isolates (Tables 1 and 2). Further, KOEHLER *et al.* (1975) reported that *A. parasiticus* isolates generally produced a greater amount of aflatoxins than *A. flavus*.

The range of aflatoxin production by *A. flavus* isolated from poultry, cattle, and camel and cattle was 5.7-110, 4.4-9.0, and 7.0-28.5 ppm, respectively. From poultry feedstuff samples, *A. parasiticus* produced aflatoxins in the range 212.5-232.4 ppm. In Pakistan, SALEEMI *et al.* (2010) studied the mycoflora of poultry feed and mycotoxin-producing potential of *Aspergillus* species. They reported that the toxigenic fungi content among *Aspergillus* isolates was 73.58%, and that of aflatoxigenic isolates of *A. flavus* and *A. parasiticus* was 83.33% and 85.71%, respectively. Further, they recorded that, among toxigenic *A. flavus* isolates (10/12), six produced four aflatoxins (AFB1, AFB2, AFG1, and AFG2), two produced AFB1, AFB2, and AFG1, one produced AFB1, AFB2, and AFG2, and one produced AFB1 and AFB2. Among aflatoxigenic isolates of *A. parasiticus* (6/7), five produced four aflatoxins (AFB1, AFB2, AFG1, and AFG2) while one produced three (AFB1, AFB2, and AFG1).

The production range of aflatoxins from four isolates (TUH212, 221, 222, and 225) of *A. parasiticus* retrieved from cattle feed samples was 143.6-271.3 ppm. Further, two isolates (TUHT216 and 220) of *A. parasiticus* isolated from cattle and camel feed samples contained 195.5 and 211.2 ppm of aflatoxins (Table 1). Among isolates of *A. flavus* collected from Taif samples, TUHT53 showed the lowest aflatoxin potential (7.0 ppm) and TUHT44 showed the highest (106.8 ppm). Isolates TUHT185 and TUHT180 from feed samples collected from Jeddah showed the lowest (5.7 ppm) and highest (33.0 ppm) levels of aflatoxins, respectively (Table 1). For *A. flavus* isolates retrieved from feed samples collected from Makkah, TUHT117 and TUHT121 showed the lowest (5.0 ppm) and the highest (110 ppm) aflatoxin levels, respectively. The results shown in Table 2 indicated that, from *A. parasiticus* isolates, the lowest aflatoxin producer was TUHT212 (143.6 ppm), while the highest production was recorded in isolate TUHT222 (269.5 ppm). Data from different geographic areas demonstrated a great variability in the mycotoxin-producing potential of *A. flavus* and closely related species (HORN and DORNER, 1999). These results are in accordance with previous reports showing that these two species have the ability to produce both B and G aflatoxins (PITT and HOCKING, 1997; KUMEDA *et al.*, 2003; GHIASIAN *et al.*, 2004). In Al-

Table 1 - Total aflatoxins (PPM) and aflatoxigenic genes detected in 64 strains of *Aspergillus flavus* isolates collected from feedstuff samples.

Strains code	Source of isolation	Location	Total AFs (PPM)	aflR	omt-A	ver-1	nor-1
TUHT43	Poultry	Taif	N.D.	+	+	+	+
TUHT44	Poultry	Taif	106.8	+	+	+	+
TUHT46	Poultry	Taif	7.8	+	+	+	+
TUHT47	Poultry	Taif	N.D.	+	+	+	+
TUHT53	Poultry	Taif	7.0	+	+	+	+
TUHT59	Poultry	Taif	N.D.	+	-	+	+
TUHT63	Poultry	Taif	N.D.	-	+	+	+
TUHT84	Poultry	Taif	N.D.	-	-	-	+
TUHT85	Poultry	Taif	N.D.	+	-	-	-
TUHT86	Poultry	Taif	11.0	+	+	+	+
TUHT87	Poultry	Taif	N.D.	+	+	-	+
TUHT89	Cattle	Taif	N.D.	-	-	-	-
TUHT91	Cattle	Taif	10.0	+	+	+	+
TUHT92	Camel & cattle	Taif	N.D.	+	+	-	-
TUHT93	Poultry	Taif	16.0	+	+	+	+
TUHT94	Poultry	Taif	28.4	+	+	+	+
TUHT98	Camel & cattle	Taif	N.D.	+	-	-	-
TUHT99	Cattle	Taif	19.0	+	+	+	+
TUHT100	Poultry	Makkah	N.D.	-	+	-	-
TUHT104	Poultry	Makkah	N.D.	+	+	-	+
TUHT106	Poultry	Makkah	N.D.	-	+	-	+
TUHT107	Poultry	Makkah	8.9	+	+	+	+
TUHT108	Poultry	Makkah	N.D.	-	+	-	+
TUHT109	Poultry	Makkah	20.0	+	+	+	+
TUHT110	Poultry	Makkah	12.0	+	+	+	+
TUHT111	Cattle	Makkah	13.0	+	+	+	+
TUHT115	Camel & cattle	Makkah	N.D.	+	+	-	+
TUHT116	Cattle	Makkah	19.0	+	+	+	+
TUHT117	Cattle	Makkah	4.4	+	+	+	+
TUHT118	Cattle	Makkah	15.0	+	+	+	+
TUHT119	Camel & cattle	Makkah	N.D.	-	+	+	-
TUHT120	Camel & cattle	Makkah	28.5	+	+	+	+
TUHT121	Poultry	Makkah	110.0	+	+	+	+
TUHT123	Poultry	Makkah	N.D.	+	+	-	-
TUHT124	Poultry	Makkah	6.6	+	+	+	+
TUHT126	Poultry	Makkah	13.2	+	+	+	+
TUHT152	Poultry	Makkah	N.D.	+	-	-	-
TUHT154	Poultry	Makkah	8.5	+	+	+	+
TUHT155	Poultry	Makkah	N.D.	+	-	-	+
TUHT156	Poultry	Makkah	12.6	+	+	+	+
TUHT157	Cattle	Makkah	N.D.	+	-	-	-
TUHT158	Cattle	Makkah	7.3	+	+	+	+
TUHT160	Poultry	Jeddah	N.D.	-	+	-	+
TUHT112	Horses	Jeddah	N.D.	+	+	-	+
TUHT161	Poultry	Jeddah	N.D.	+	+	+	+
TUHT163	Poultry	Jeddah	N.D.	+	+	-	-
TUHT164	Poultry	Jeddah	N.D.	+	-	+	-
TUHT165	Camel & cattle	Jeddah	7.0	+	+	+	+
TUHT166	Camel & cattle	Jeddah	N.D.	-	+	-	+
TUHT168	Horses	Jeddah	6.1	+	+	+	+
TUHT172	Camel & cattle	Jeddah	N.D.	+	+	-	-
TUHT173	Camel & cattle	Jeddah	N.D.	+	+	+	+
TUHT174	Camel & cattle	Jeddah	7.5	+	+	+	+
TUHT176	Cattle	Jeddah	N.D.	-	+	+	-
TUHT177	Cattle	Jeddah	14.0	+	+	+	+
TUHT180	Poultry	Jeddah	33.0	+	+	+	+
TUHT181	Poultry	Jeddah	21.0	+	+	+	+
TUHT185	Poultry	Jeddah	5.7	+	+	+	+
TUHT186	Camel & cattle	Jeddah	8.0	+	+	+	+
TUHT187	Camel & cattle	Jeddah	N.D.	-	-	-	+
TUHT188	Camel & cattle	Jeddah	18.2	+	+	+	+
TUHT189	Camel & cattle	Jeddah	9.13	+	+	+	+
TUHT190	Camel & cattle	Jeddah	N.D.	+	-	-	+
TUHT193	Camel & cattle	Jeddah	14.3	+	+	+	+
Total			33	53	53	42	51

Table 2 - Total aflatoxins (PPM) and aflatoxigenic genes detected in *Aspergillus parasiticus* isolates collected from feedstuff samples.

Strains code	Source of isolation	Location	Total	aflR	omt-A	ver-1	nor-1
AFs (PPM)							
TUHT226	Cattle	Taif	N.D.	+	+	-	+
TUHT227	Cattle	Taif	N.D.	+	+	+	+
TUHT228	Cattle	Jeddah	N.D.	-	+	-	+
TUHT229	Poultry	Jeddah	N.D.	+	+	+	-
TUHT26	Poultry	Taif	212.7	+	+	+	+
TUHT211	Poultry	Taif	232.4	+	+	+	+
TUHT212	Cattle	Makkah	143.6	+	+	+	+
TUHT213	Poultry	Makkah	231.2	+	+	+	+
TUHT214	Poultry	Makkah	224.4	+	+	+	+
TUHT215	Poultry	Jeddah	212.5	+	+	+	+
TUHT216	Camel & cattle	Jeddah	195.4	+	+	+	+
TUHT219	Cattle	Taif	265.5	+	+	+	+
TUHT220	Camel & cattle	Taif	211.2	+	+	+	+
TUHT221	Cattle	Makkah	271.3	+	+	+	+
TUHT222	Cattle	Makkah	269.5	+	+	+	+
TUHT223	Poultry	Jeddah	227.2	+	+	+	+
TUHT225	Cattle	Jeddah	269.7	+	+	+	+
Total (17 Isolates)			**13**	**16**	**17**	**15**	**16**

geria, RIBA *et al.* (2010) determined the aflatoxin-producing capacity of 150 *A. flavus* isolates collected from wheat and its derivatives in 2004 and 2006, and the results showed that 72% of the strains produced aflatoxins. These strains produced amounts of AFB1 in the range 12.1-234.6 µg/g of CYA medium.

The results of the present study indicate that the aflatoxigenic species of *Aspergillus* vary in their aflatoxin potential according to the substrate and environmental factors. These results are in agreement with those reported by ABBAS *et al.* (2005).

Detection of some of aflatoxin biosynthesis genes in *Aspergillus* species

The production of aflatoxin involves a complex biosynthetic pathway consisting of at least 25 genes (YABE *et al.*, 1999; CRISEO *et al.*, 2001a, BHATNAGAR *et al.*, 2003; YU *et al.*, 2004; SCHERM *et al.*, 2005). All of the identified biosynthesis-related genes are located within a 75 kb DNA region in both *A. parasiticus* and *A. flavus*, and their relative positions in the cluster of both fungal species are similar (YU *et al.*, 2000; EHRLICH *et al.*, 2005). PCR was used for the detection of aflatoxigenic aspergilli based on the intermediated enzymes, including norsolorinic acid reductase encoding gene *nor-1*, the versicolorina dehydrogenase encoding gene *ver-1*, the sterigmatocystin 0-methyl transferase encoding gene *omt-1*, and the regulatory gene *aflR* (ERAMI *et al.*, 2009).

Representative aflatoxigenic and non-aflatoxigenic *A. flavus* and *A. parasiticus* isolates were subjected for detection of aflatoxin biosynthesis genes.

Detection of aflatoxin biosynthesis genes in *A. flavus* isolates

PCR was applied using four sets of primers for different genes involved in the aflatoxin biosynthetic pathway. Bands of fragments of *aflR*, *omt-1*, *ver-1*, and *nor-1* genes were visualized at 1032 bp, 797 bp, 537 bp, and 400 bp, respectively (Fig. 1). All examined *A. flavus* isolates yielded different DNA banding patterns with a number of bands ranging from zero to four (Tables 1 and 2).

Table 1 outlines the total aflatoxin and aflatoxigenic genes (*aflR*, *omt-A*, *ver-1*, and *nor-1*) detected in 64 strains of aflatoxigenic and non-aflatoxigenic *A. flavus* isolates collected from feedstuff samples. *A. flavus* isolates were represented by 35 isolates from poultry feed samples, 16 from camel and cattle feed, 11 from cattle feed, and two from horse feed. Thirty-eight out of 64 (59.4%) *A. flavus* isolates contained all four aflatoxin biosynthesis genes; among them 21 isolates were retrieved from poultry feedstuff samples, eight from camel and cattle feed, eight from cattle feed, and one from horse feed (Table 1). This result is in agreement with CRISEO *et al.* (2001a), who used specific PCR-based methods to prove that aflatoxigenic *A. flavus* isolates always contain the complete gene set.

Among the 38 isolates tht showed the presence of all four targeted genes, two isolates (TUHT43 and 47) were not aflatoxigenic. Therefore, this result indicated clearly that the presence of the four tested genes is not a sufficient marker for the differentiation between aflatoxigenic and non-aflatoxigenic isolates. Other studies (FLAHERTY and PAYNE, 1997; CHANG *et al.*, 1999a,b; 2000, CARY *et al.*, 2002; TAKAHSHI *et*

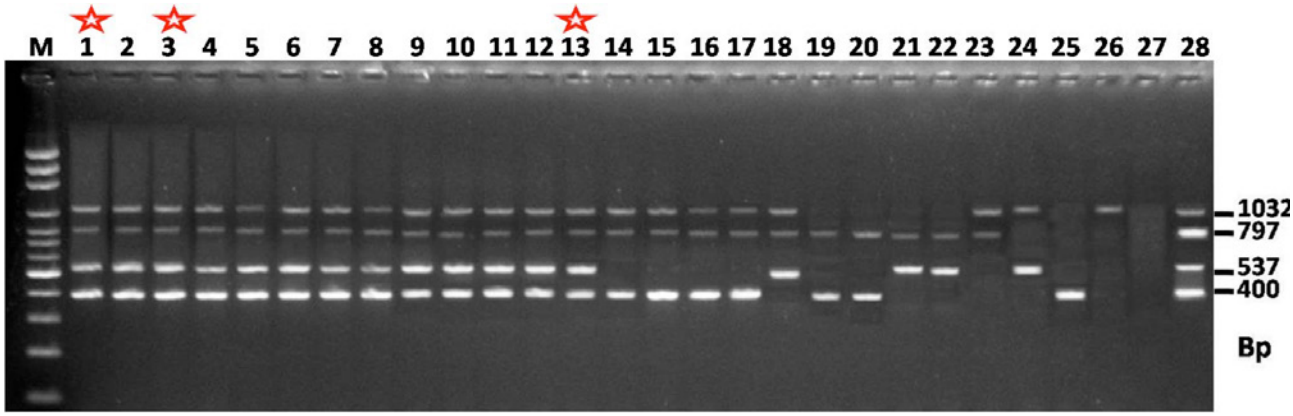

Fig. 1 - Aflatoxin biosynthesis genes amplifications. Lanes 1 - 7, *Aspegillus flavus* (TUHT 43, 44, 47, 91, 168, 188 and 219); Lanes 8 - 13, *A. parasiticus* (TUHT 26, 212, 216, 221, 223 and 227); lanes 14 -16, *A. flavus* (TUHT 87, 104, 115), lane 17 *A. parasiticus* (TUHT 226), lane 18 *A. parasiticus* (TUH 229); Lane 19, *A. flavus* (TUHT 160); Lane 20, *A. parasiticus* (TUHT 228); Lane 21 & 22, *A. flavus* (TUHT 119 & 176); Lane 23, *A. flavus* (TUHT 163); Lanes 24; *A. flavus* (TUHT 164); lane 25 *A. flavus* (TUHT84); lane 26 *A. flavus* (TUHT157); lane, 27 negative control and lane 28, positive control.
Asterisked lanes were non aflatoxigenic isolates.

al., 2002; EHRLICH *et al.*, 2003) have suggested that regulation of aflatoxin biosynthesis in *Aspergillus* spp. involves a complex pattern of positive and negative acting transcriptional regulatory factors affected by environmental and nutritional parameters. Furthermore, the lack of aflatoxin production apparently does not need to be related only to an incomplete pattern obtained in PCR-based detection. Different mutations may be responsible for the inactivation of aflatoxin biosynthetic pathway genes in other *A. flavus* strains (GEISEN, 1996).

Six isolates (9.4% of the tested isolates) with three gene amplicons were not aflatoxigenic (Table 3). From these, four, one, and one isolates were retrieved from poultry, camel and cattle, and horse feeds, respectively. Twelve isolates (18.8% of the tested isolates; six from poultry, five from camel and cattle, and one from cattle), contained two gene amplicons and seven isolates (10.9%) contained one gene amplicon (Table 3). On the other hand, one non-aflatoxigenic isolate (TUHT89) showed no bands, indicating a deletion of the targeted genes in this isolate. CRISEO *et al.* (2001a) proved that non-aflatoxigenic isolates of *A. flavus* were 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. Further, CRISEO *et al.* (2001b) reported that aflatoxin biosynthesis in *A. flavus* is strongly dependent on the activities of regulatory proteins and enzymes encoded by the four genes *aflR*, *nor-1*, *ver-1*, and *omt-A*. GHERBAWY *et al.* (2012) reported on the presence of a complete set of these genes in seven aflatoxigenic isolates of *A. flavus* retrieved from date palm.

The frequencies of the four aflatoxin biosynthesis genes *aflR*, *omt-A*, *ver-1*, and *nor-1*, in the tested isolates were 53, 53, 42, and 51, respectively (Table 1). CRISEO *et al.* (2008) used 134 of non-aflatoxin producing strains of *A. flavus* isolated from food, feed, and officinal plants to study the different genes involved in the aflatoxin biosynthetic pathway. Their results indicated that the *nor-1* gene was the most representative (88%) of the four aflatoxin structural assayed genes, followed by *ver-1* and *omt-A*, which were found at the same frequency (70.1%). A lower incidence (61.9%) was observed for *aflR*. Further, CRISEO *et al.* (2008) demonstrated that a high number of aflatoxin non-producing strains (61.9%) contain the *aflR* gene. This could impair the use of *aflR* to identify aflatoxigenic aspergilli. Five out of ten *A. flavus* isolates were not aflatoxin producers (SCHERM *et al.*, 2005),

Table 3 - Origin and genetic patterns of 64 aflatoxigenic *Aspergillus flavus* isolates collected from feedstuff samples in this study. Values in brackets are percentages of the total samples analyzed.

Sample name	No isolates	Complete set	Three bands	Two bands	One band	Zero band
Poultry	35	21	4	6	4	-
Camel & cattle	16	8	1	5	2	-
Cattle	11	8	-	1	1	1
Horses	2	1	1	-	-	-
Total	**64 (100)**	**38 (59.4)**	**6 (9.4)**	**12 (18.8)**	**7 (10.9)**	**1 (1.6)**

indicating that the frequencies of occurrence of *aflR* and *omt-A*, *ver-1*, and *nor-1* genes were 8, 5, 9, and 5, respectively.

Detection of some of aflatoxin biosynthesis genes in *A. parasiticus* isolates

Seventeen *A. parasiticus* isolates collected from different feedstuff samples from various cities in Saudi Arabia were examined for the presence of aflatoxin biosynthesis genes using a specific primer set as mentioned above. The results indicated the presence of four bands for *aflR*, *omt-1*, *ver-1*, and *nor-1* genes at 1032 bp, 797 bp, 537 bp, and 400 bp, respectively (Fig. 1). All aflatoxigenic and non-aflatoxigenic isolates examined yielded different DNA banding patterns with the number of bands ranging from 2 to 4 (Tables 2 and 4).

Table 2 shows the total aflatoxin and aflatoxigenic genes detected in *A. parasiticus* isolates collected from three different feedstuff samples (poultry, camel and cattle, and cattle). Thirteen out of 17 *A. parasiticus* isolates were aflatoxigenic. The frequencies of occurrence of *aflR*, *omt-1*, *ver-1*, and *nor-1* genes in *A. parasiticus* isolates were 16 (94.1%), 17 (100%), 15 (88.2%), and 16 (94.1%), respectively. Gherbawy *et al.* (2014) reported that *omt-A* was the most prevalent gene in *A. flavus* and *A. parasiticus* isolated from chili samples collected from Taif city (Saudi Arabia). Further, their results indicated that this gene was recovered from 27 out of 30 *A. flavus* isolates and two isolates of *A. parasiticus*, while *nor-1, aflR*, and *ver-1* genes were recovered from 25, 26, and 24 isolates of aflatoxigenic and non-aflatoxigenic isolates of *A. flavus*. Out of seven *A. parasiticus* isolates collected from poultry feedstuff samples, 6 (85.7%) contained four genes, while two (14.3%) showed the amplicons of three genes. The two *A. parasiticus* isolates collected from camel and cattle feedstuff samples showed a complete set of the targeted genes (Tables 2 and 4). Amplification of the four targeted genes in eight *A. parasiticus* isolates collected from cattle feedstuff samples showed that six (75%) had the four genes and one (12.5%) contained three genes (Tables 2 and 4). Further, one isolate contained two genes. GEISEN (1996) reported the presence of the abovementioned genes from two isolates of *A. parasiticus*. Additionally, SCHERM *et al.* (2005) indicated the presence of a complete set of genes (*aflR*, *omt-1*, *ver-1*, and *nor-1* genes) in three isolates of *A. parasiticus*.

The findings herein showed the presence of four targeted genes in all aflatoxigenic isolates of *A. parasiticus* and in one (TUHT229) non-aflatoxigenic isolate. Further, all non-aflatoxigenic isolates were missing one or more of the targeted genes. RASHID *et al.* (2008) studied the presence of *aflR*, *omt-1*, *ver-1*, and *nor-1* genes in 35 *A. parasiticus* isolates from stored wheat grains in Pakistan. Their results revealed that only one isolate showed the complete set of genes. Additionally, *omt-1*, *ver-1*, and *nor-1* genes appeared in 8, 10, and 13 isolates. Deletion of *aflR* in *A. parasiticus* abolishes the expression of other aflatoxin pathway genes (CARY *et al.*, 2000). Finally, the regulation of aflatoxin biosynthesis genes in *Aspergillus* spp. is affected by environmental and nutritional parameters (FLAHERTY and PAYNE, 1997; CHANG *et al.*, 2000; CARY *et al.*, 2002; TAKAHSHI *et al.*, 2002; EHRLICH *et al.*, 2003).

Genetic diversity among *A. flavus* strains isolated from feedstuff samples

Sixty-four aflatoxigenic and non-aflatoxigenic isolates of *A. flavus* represented different sources of isolation and different locations were used in this part. Using the ITS region of rRNA sequencing results and amplification of some aflatoxin biosynthesis genes, the genetic diversity among those strains was studied.

Using ITS sequencing results of 64 isolates of *A. flavus*, a neighbor joining tree was constructed (Fig. 2). The population of *A. flavus* split into several clades and sub-clades; the bootstrap values for these clades and sub-clades ranged from 1 to 100, indicating a high heterogeneity in this population. Further, the clustering system did not correlate with the type of sample or its location. For example, *A. flavus* isolate TUTH157 (isolated from cattle feedstuff sample collected from Makkah) clustered together with isolate TUTH63 (isolated from poultry feedstuff sample collected from Taif) in one sub-clade with a 98 bootstrap value. Additionally, isolates TUTH154 and TUTH193 constituted one sub-clad with a 69 bootstrap value, although the first one was

Table 4 - Origin and genetic patterns aflatoxigenic *Aspergillus parasiticus* isolates collected from feedstuff samples. Values in brackets are percentages of the total samples analyzed.

Sample name	No isolates	Complete set	Three bands	Two bands
Poultry	7	6	1	-
Camel & cattle	2	2	-	-
Cattle	8	6	1	1
Total	**17 (100)**	**14 (82.4)**	**2 (11.8)**	**1 (5.9)**

isolated from poultry feedstuff samples from Makkah and the second from cattle and camel feedstuff samples from Jeddah (Fig. 2 and Table 1). Therefore, clustering according to the ITS sequencing results did not indicate any relationship among the isolate clustering system and their geographical distributions and even the sources of isolation. Aflatoxigenic isolates spread all over the constricted phylogentic tree without separation of the clades into toxigen-

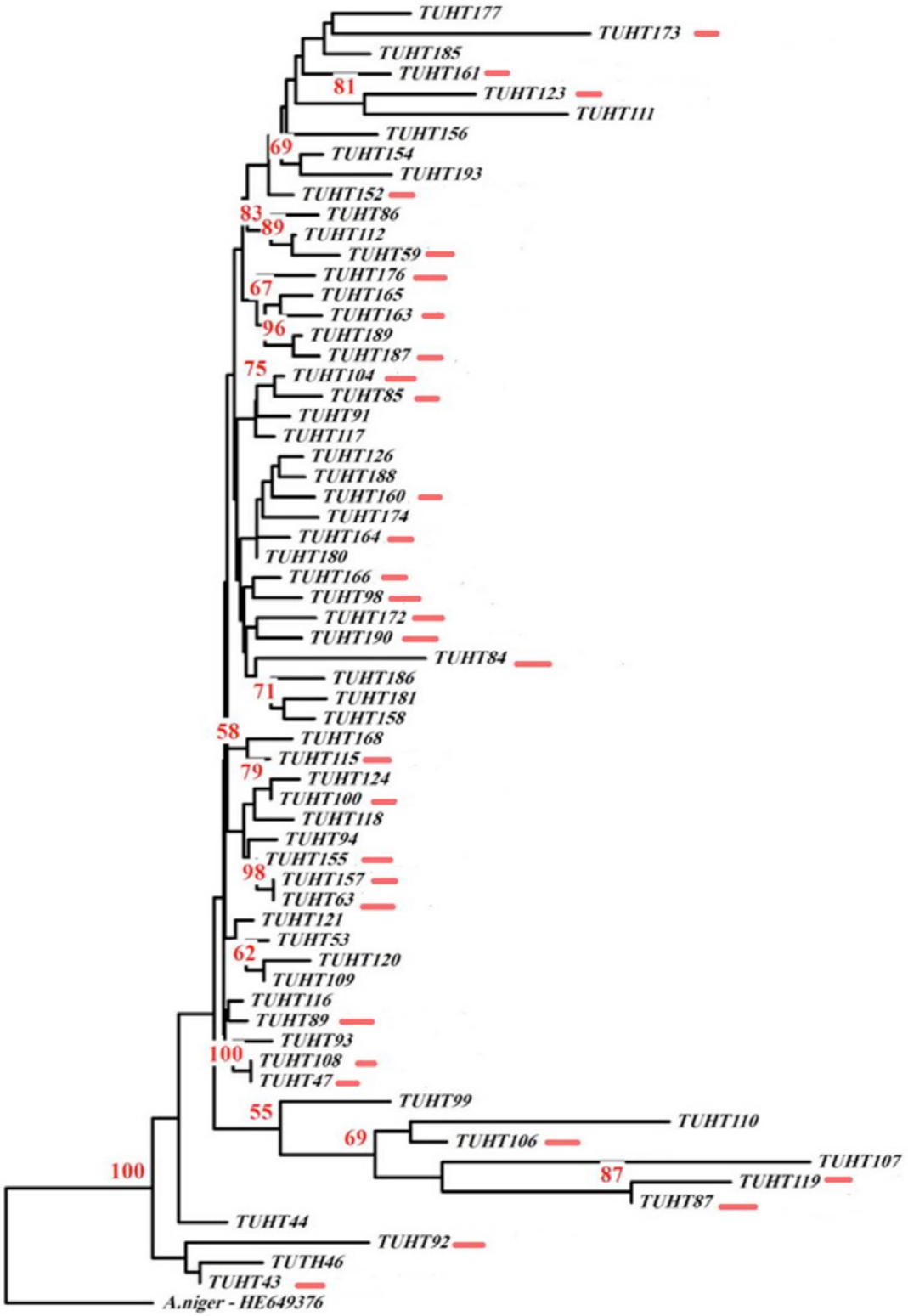

Fig. 2 - Phylogenetic tree based on the internal transcribed spacer (ITS) region of rRNA of aflatoxigenic and non aflatoxigenic 64 isolates of *Aspergillus flavus*. The tree was constructed by neighbor-joining algorithm using maximum composite likelihood model. Bootstrap factors less than 55 were not shown. The tree was rooted with *Aspergillus niger* [HE649376] as the out-group. Red rods indicated non aflatoxigenic isolates.

ic and non-toxigenic. For example, aflatoxigenic isolate TUHT189 clustered with non-aflatoxigenic isolate TUHT187 with a 96 bootstrap value (Fig. 2). Since the clustering system was based on ITS sequencing results, with non-functional spacers, there is no correlation between clustering system and toxin production. The present results show that isolates identified as *A. flavus* had a polyphyletic origin, supporting the genetic heterogeneity of *A. flavus* as previously dem-

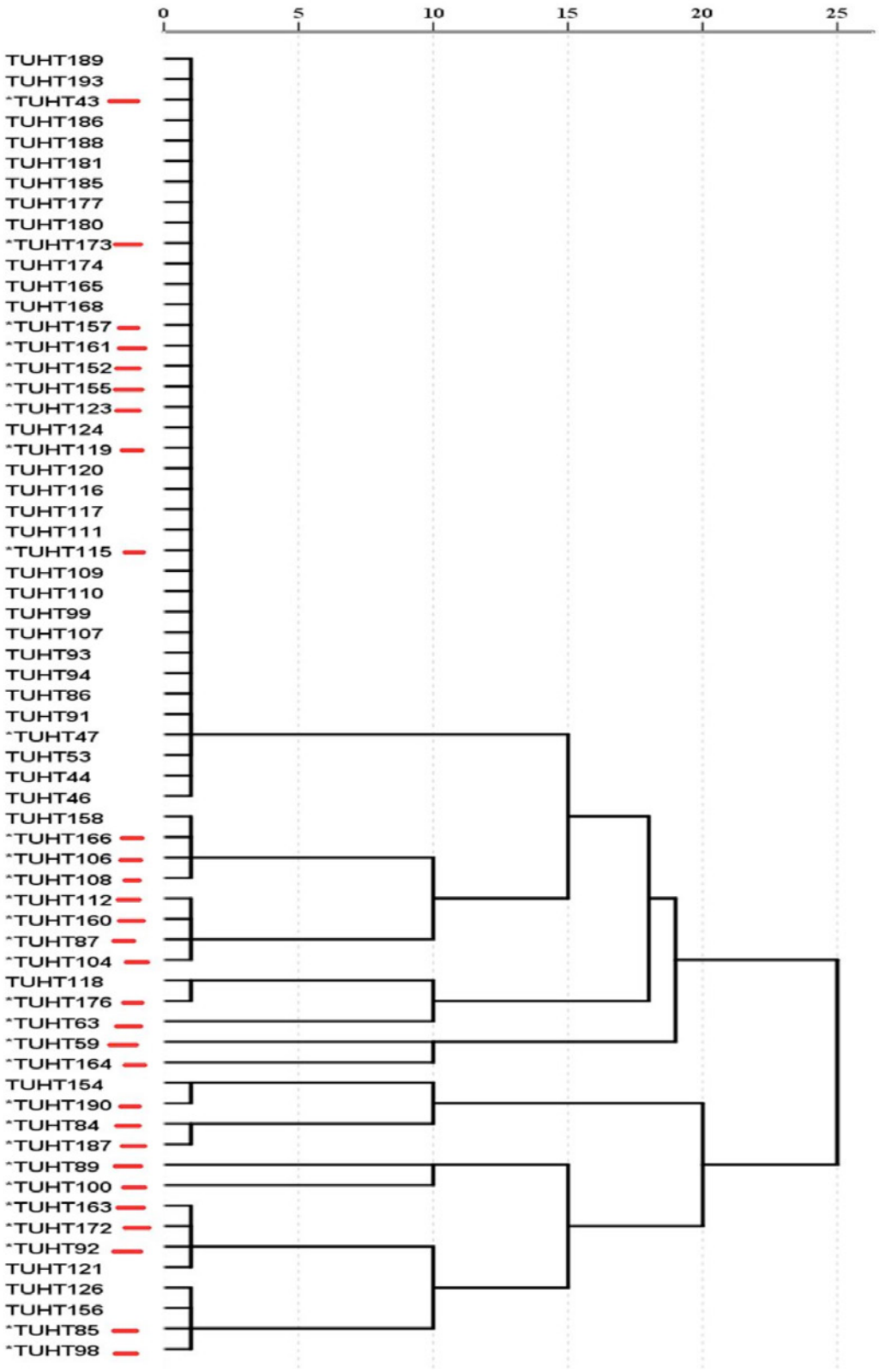

Fig. 3 - The hierarchical cluster analysis using average linkage between groups form *Aspergillus flavus* isolates based on amplification of aflatoxins biosynthesis genes. Red bars indicated non aflatoxigenic species.

onstrated by other studies (GEISER *et al.*, 1998, 2000; VAN DEN BROEK *et al.*, 2001; CHANG *et al.*, 2007; GONCALVES *et al.*, 2012).

The genetic diversity among *A. flavus* isolates was studied using the results of amplification of some aflatoxin biosynthesis genes. The results were subjected to hierarchical cluster analysis using average linkage between groups to construct a dendrogram showing the correlation between the isolates (Fig. 3). *A. flavus* isolates did not follow any rule in their clustering system. For example, aflatoxigenic isolates TUHT121 and TUHT126 (isolated from a poultry feedstuff sample collected from Makkah) and non-aflatoxigenic isolates TUHT85 (poultry feedstuff samples from Taif) and TUHT98 (camel and cattle feeds from Taif) were clustered together as shown in Fig. 3. On the contrary, TUHT112 (horse feeds from Jeddah), TUHT160 (poultry feeds from Jeddah), TUHT87 (poultry feeds from Taif), and TUHT104 (poultry feeds from Makkah) were non-aflatoxigenic isolates clustered together (Fig. 3). Generally, these results indicate that the presence or absence of PCR products for the targeted aflatoxin biosynthesis genes was not correlated with the type of feedstuff or the location of sample collection. Previous authors (GEISER *et al.*, 1998, 2000; MOORE *et al.*, 2009, GONCALVES *et al.*, 2012), found that the aflatoxin cluster genes were useful tools for phylogenetic studies in the section *Flavi*.

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