Australian Journal of Crop Science AJCS
AJCS 7(11):1707-1713 (2013) ISSN:1835-2707

A polyphasic method for the identification of aflatoxigenic *Aspergillus* species isolated from Camel feeds

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Abstract

The main goal of our study was to identify *Aspergillius* spp. isolates by polyphasic taxonomic techniques. Differential culture media, biochemical and molecular characterization were applied to 21 isolates of *Aspergillus flavus* and *Aspergillus niger* from Saudi Arabia camel feeds. Six aflatoxin producing culture media were used for characterizing and identifying aflatoxigenic isolates. The blue fluorescent ring visible under UV light which indicates the ability to produce aflatoxin, by aflatoxinogenic strains was not observed for any of the tested non aflatoxigenic isolates. Biochemical characterization involving the screening of the isolates for five aflatoxins was also performed. As found, most isolates were capable of producing detectable levels of both B and G type's aflatoxins (AFs) and maltoryzine, although 4 of the 7 *A. niger* isolates failed to produce any detectable amount of AFs. PCR was performed using one set of primers that specifically targets the aflatoxin regulatory gene (*aflR*) involved in aflatoxin biosynthetic pathway as well as four specific primers for *A. flavus* and *A. niger*. The presence of the *aflR* gene did not correlate with aflatoxigenicity. Most of the fungi belonging to *A. flavus* group reacted positively with aflR primers that cover the region from 540 to 1338 of aflatoxin regulatory gene with product size of 798 base pairs (bp). All *A. flavus* isolates had positive PCR results using the primer pair FLA1-FLA. A unique DNA fragment of the expected 500-bp size was amplified in all *A. flavus* isolates, while no PCR products were visualized in other *Aspergillus* species or members belong to other fungal genera. Using the primer pairs OMt1R-OMt1F, a single fragment of about 1232 bp *A. niger* isolates was amplified but did not amplify with DNA extracted from other *Aspergillus* species or species belonging to other fungal genera. Detection and quantification of these two important aflatoxin-producing Aspergilli could provide important information to predict the aflatoxin profiles which may be present in the feed matrix.

Keywords: *Aspergillus flavus*; aflatoxin genes; camel feed; maltoryzine; Saudi Arabia.
Abbreviations: aflR_aflatoxin regulatory; AFs_Aflatoxins; ADM_Aspergillus differential medium; AFPA_*Aspergillus flavus* and *parasiticus* Agar; CA_Coconut agar; CZ_Czapek agar; HPLC_high performance liquid chromatography; PDA_Potato Dextrose Agar; TLC_thin-layer chromatography; YES_yeast extract sucrose agar.

Introduction

Some feed-borne *Aspergillus* species are capable of producing aflatoxins (AFs), which are toxic secondary metabolites. Aflatoxins have a high potential to contaminate animal feeds formulated and stored in environments favourable for the growth of fungi (Kumar et al., 2008; Krnjaja et al., 2009). Aflatoxins, particularly in animal feeds and feed components, pose serious public health risks as they are highly toxic (Yaling et al., 2008). The International Agency for Research on Cancer classified aflatoxin B1 (AFB1) as a Group 1A human carcinogen (IARC, 1993). There are at least 16 structurally related AFs characterized, and among them, only four i.e. AFB1, AFB2, AFG1, and AFG2 are considered important natural contaminants of crops and other agricultural supplies (Leontopoulos et al., 2003). In Saudi Arabia, imported maize samples collected from various farms in the Riyadh region of Saudi Arabia were found to contain high levels of AFs (Ewaidah, 1992). In the same country, fodder samples were analyzed for fungi and mycotoxins especially AFs, ochratoxin A (OTA) and zearalenone (ZEA). Natural feeds (grains or bran) were more contaminated than compound feeds, which was under the regulatory limitation (Bokhari, 2010). In the Riyadh region, AFs have caused the deaths of over 6000 camels within a year. Losses were estimated at millions of Riyals and as a result, laid a foundation to alleviate the problem of mycotoxins in feed (Almoammar, 2012). The conventional method used for species identification based on morphological and biochemical characters is time-consuming and not always straight-forward, however, molecular methods can be suitable (Rodrigues et al., 2009). Misidentification can occur because some aflatoxigenic fungi may be unsuccessfully characterized or because considerable proficiency is required. The current methods used mainly for detection of *A. flavus* and *A. niger* include conventional methods such as *Aspergillus* differential medium (ADM). These methods are based on the use of complex media to detect the natural fluorescence of AFs released by the growing mycelium (Hara et al., 1974; Fente et al., 2001; Maragos et al., 2008). Molecular techniques have been used to ascertain the aflatoxigenicity of *A. flavus* and *A. niger* fungi in food and feed (Geisen, 1996; Shapira et al., 1996; Mayer et al., 2003; Somashekar et al., 2004; Abdel-Hadi et al., 2010). In this respect, a number of specific primers have been designed for several genes involved in the biosynthetic

pathways of AFs i.e. *afl, nor, omt, ord, tub* and *ver* (Scherm et al., 2005; Rodrigues et al., 2009). The existence of four genes: *nor A, ver 1, omt A,* and *avf A* in seven *A. flavus* toxigenic isolates collected from Saudi Arabia was evaluated using quadruplex polymerase (Gherbawy et al., 2012). Manonmani et al. (2005) using an indigenously specific primer pair for the aflatoxin regulatory (*aflR*) gene evaluated the presence of aflatoxigenic fungi in feed and foodstuffs. Variability in AF production potential of *A. flavus* isolates has been reported (Karthikeyan et al., 2009). The identification of toxigenic fungi is very important to prevent toxin contamination of animal feed during both pre-harvest and post-harvest. Currently, there is an additional need for these methods to be applied in developing countries to screen large numbers of aflatoxigenic species. Such a need enabled us to pay attention on polyphasic methods for aflatoxigenicity testing methods (Rodrigues et al., 2009).The purpose of this research work was to apply mycological, biochemical and molecular methods for the detection of aflatoxigenic fungi present in camel feeds from Saudi Arabia.

Results

Aspergillus differential media

A visible beige ring withoutlight was observed in cultures of aflatoxigenic fungi (Fig.1). Visualization of a blue fluorescent ring surrounding aflatoxigenic colonies under UV light was also possible. The aflatoxigenic *A. flavus* and *A. niger* isolates showed fluorescence in PDA+NaCl and APA media after 6 days of incubation (Table 3). The diameter of the beige ring and the intensity of its fluorescence under UV increased over time with the maximum observed on day four.

Screening of Aspergillus species for aflatoxin production

Most isolates were capable of producing detectable levels of both B and G types AFs as well as maltoryzine, however, four of the seven *A. niger* isolates failed to produce any detectable amount of aflatoxins. In overall, the other three *A. niger* isolates were the lowest producers of AFs, followed by *A. flavus.* The highest amount of total AF (17.8 µg/kg) was recovered from a culture of *A. flavus* (isolate 4). The highest amount of aflatoxin B1 production (8.2 µg/kg) was obtained from *A. flavus* (isolate 5). The highest amount of aflatoxin maltoryzine production (1.4 µg/kg) was obtained from *A. niger* (isolate 1). Three of the nine *A. niger* isolates tested failed to produce any detectable amount of AFs. From Table 2, several of the *A. flavus* isolates produced large quantities of AFs than any of the *A. niger* isolates. Limit of detection (LOD) is 0.1µg/kg for all AFs.

Identities of aflatoxigenic isolates following PCR assay

The specificity of AflR1-AflR2 primers was analyzed by conventional PCR in a number of *A. flavus* and *A. niger* isolates from different feed products as indicated above. When genomic DNA from *A. flavus* was used, a single fragment of about 796 bp was amplified. However, no amplification product was obtained in genomic DNA from *A. flavus* isolates and other fungal species tested (Fig. 1). The DNA of *Alternaria alternata* and *Fusarium semitectum* were also subjected to PCR using AflR primers, but no amplicons were found. A pair of primers, *Anigf* and *Anigr*, specific to *A. niger* was designed on the basis of the sequence alignment. All *Aspergillus* isolates listed in Table 2 were tested for amplification using the primer pair *OMt1R-OMt1F*. A single fragment of about 1232 bp was only amplified when genomic DNA from *A. niger* isolates was used (Fig. 2). The DNA of *A. alternata* and *F. semitectum* were also subjected to PCR using *Anig* primers, but no amplicons were obtained. *Aspergillus* isolates listed in Table 2 were tested for amplification using the primer pair *FLA1* and *FLA2*. A single fragment of about 500 bp was only amplified when genomic DNA from *A. flavus* isolates was used (Table 1), but not from other Aspergilli, including *A. niger, A. ochraceus,* and *A. carbonarious*. No PCR product was obtained with genomic DNA from other genera. This approach of differentiating these two species would be simpler, less costly and quicker than conventional sequencing of PCR products.

Discussion

A polyphasic method consisting of cultural, chemical and molecular characterization was applied to 21 isolates of *Aspergillus* species from camel feeds originating from Saudi Arabia. This was with the aim of characterizing and identifying aflatoxigenic and non-aflatoxigenic isolates. Among the *Aspergillus* isolated from feeds, *A. flavus* was the predominant species followed by *A. niger*. These results are in harmony with some reports (Accensi et al., 2004; Rosa et al., 2006; Somashekar et al., 2004). Most isolates were capable of producing measurable levels of both B and G types AFs and maltoryzine, although four of the seven *A. niger* isolates failed to produce any detectable amounts. The other three *A. niger* isolates were the best producers of AFs over all, followed by *A. flavus.* Toxigenic property of *A. niger* isolates was confirmed by detecting AFs by high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC), respectively (Yassin et al., 2010; Gherbawy et al., 2012). A majority (77%) of *A. flavus* isolates were aflatoxigenic, the highest amount of AFB1 (7.8 µg/kg) produced by an isolate of *A. flavus*. Aflatoxigenic ability is, in fact, an unstable characteristic exhibited by strains of *A. flavu*s, and their adaptation in carbon-rich environments of certain agricultural commodities may result in loss of gene responsible for aflatoxigenicity (Perrone *et al.,* 2007). This could possibly be the case for our substrate. *A. flavus* isolates are known to produce AFB1 on various agricultural commodities (Mbah and Akueshi, 2009). These results are in agreement with Abbas *et al.* (2005) who observed greater differences in AF production by *A. flavus*. Six culture media were used to determine the aflatoxigenicity of fungal isolates without additives. According to the results mentioned above, it seems evident that the presence of a beige ring visible at first sight along with fluorescence in the surrounding colonies as examined under UV is strongly an indication of AF-producing ability of that isolate. In addition, it has been found that it is easier (especially in laboratories that lack facilities for chemical determination of AFs and more economical to first identify aflatoxigenic fungi in contaminated materials and AFs by examining fluorescence on agar than to chemically test for AFs. The detection of AFs as judged by the fluorescence of fungal colonies is not easy, because non-aflatoxigenic isolates of belonging to the *Aspergillus* genera such as *A. flavus* and *A. niger*, fluoresce under UV. Fluorescence and phosphorescence are not the only outcomes of exposure of AFs to UV light. AFB2 and AFB2 are activated by 365 nm UV light, resulting in AFB2–8,9-oxide. The naturally occurring AFs are designated as AF B1, B2, G1, and G2. B and G forms are recognized on the basis that they emit blue or green fluorescence upon exposure to ultraviolet light (Murphy et al., 2006). A yellow pigmentation has previously been associated with AF

Table 1. *Aspergillius* and other fungal species screened and scored for presence and absence of aflatoxin genes using specific primers**.**

Aspergillius species	*Aflatoxin gene specific-primers*				
	ASP1P	*ASP2P*	*AnigF/AnigR*	*AflR-1/AflR-2*	*FLA1/FLA2*
1. A. niger	+	-	+	-	-
2. A. niger	+	-	+	-	-
3. A. niger	+	-	+	-	-
4. A. niger	+	-	+	-	-
5. A. niger	+	-	+	-	-
6. A. niger	+	-	+	-	-
7. A. niger	+	-	+	-	-
8. A. niger	+	-	+	-	-
9. A. niger	+	-	+	-	-
10. A. niger	+	-	+	-	-
11. A. niger	+	-	+	-	-
12. A. niger	+	-	+	-	-
13. A. niger	+	-	+	-	-
14. A. niger	+	-	+	-	-
15. A. flavus	-	-	-	+	+
16. *A. flavus*	-	-	-	+	+
17. *A. flavus*	-	-	-	+	+
18. *A. flavus*	-	-	-	+	+
19. *A. flavus*	-	-	-	+	+
20. *A. flavus*	-	-	-	+	+
21. *A. flavus*	-	-	-	+	+
22. *A. flavus*	-	-	-	+	+
23. *A. flavus*	-	-	-	+	+
24. *A. flavus*	-	-	-	+	+
25. *A. flavus*	-	-	-	+	+
26. *A. flavus*	-	-	-	+	+
27. *A. flavus*	-	-	-	+	+
28. *A. flavus*	-	-	-	+	+
Control					
29. A. niger (ASP-KSU1)	+	+	+	+	-
30. A. flavus (ASP-KSU2)	-	-	-	+	+
31. A. ochraceus (ASP-KSU8)	-	-	-	-	-
32. A. carbonarious (ASP-KSU8)	-	-	-	-	-
33. *Fusarium semitectum*	-	-	-	-	-
34. Penicillium citrinum	-	-	-	-	-
35. *Macrophomina phasolina*	-	-	-	-	-

Detection of the PCR product on 1% agarose gel: - no product detected; + PCR product detected.

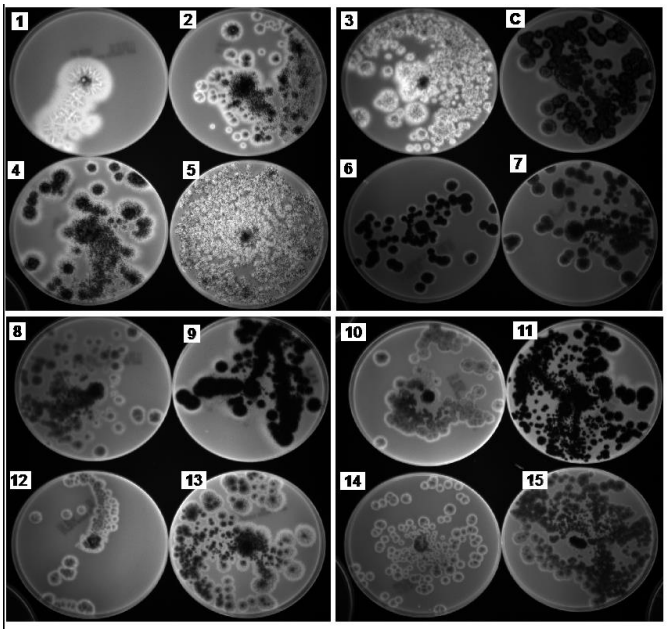

Fig 1. A aflatoxigenic isolate of *A. niger* (1-7) and *A. flavus* (8-15) visualized under visible light and under 365 nm UV light (C, Control) on Coconut agar (CA). The rim of the white ring around the colony of the aflatoxigenic isolate displays faint blue florescence.

Table 2. Production of aflatoxins and maltoryzine by *Aspergillus* spp. isolated from feed products.

No.	Isolates	Mycotoxins (µg/kg)					
		AFB1	AFB2	AFG1	AFG2	Total AF	maltoryzine
1	*Aspergillus flavus*	2.3	3.5	1.2	3.4	10.4	1.3
2	*Aspergillus flavus*	5.2	1.2	0.0	2.7	9.1	1.1
3	*Aspergillus flavus*	1.3	0.0	0.0	1.4	2.7	0.6
4	*Aspergillus flavus*	7.8	2.2	4.3	3.5	17.8	0.1
5	*Aspergillus flavus*	6.3	5.4	8.2	4.4	24.3	0.9
6	*Aspergillus flavus*	0.0	0.0	0.0	0.1	0.1	0.5
7	*Aspergillus flavus*	6.4	2.3	0.0	0.0	8.7	1.3
8	*Aspergillus flavus*	2.3	2.5	0.9	2.3	8.0	0.1
9	*Aspergillus flavus*	1.2	6.4	2.2	1.0	10.8	0.2
10	*Aspergillus flavus*	2.0	4.3	1.2	3.2	10.7	0.0
11	*Aspergillus flavus*	0.9	0.1	0.0	0.2	1.2	0.0
12	*Aspergillus flavus*	1.5	0.0	0.4	0.8	2.7	0.3
13	*Aspergillus niger*	0.0	0.0	0.0	0.0	0.0	1.4
14	*Aspergillus niger*	0.1	0.4	0.0	0.2	0.7	0.9
15	*Aspergillus niger*	0.2	0.1	0.0	0.0	0.3	1.0
16	*Aspergillus niger*	0.3	0.0	0.0	0.1	0.4	0.2
17	*Aspergillus niger*	0.2	0.0	0.2	0.2	0.6	0.0
18	*Aspergillus niger*	0.1	0.1	0.0	0.0	0.2	0.6
19	*Aspergillus niger*	0.1	0.0	0.1	0.2	0.4	0.1
20	*Aspergillus niger*	0.0	0.0	0.0	0.0	0.0	0.8
21	*Aspergillus niger*	0.0	0.0	0.0	0.0	0.0	0.0

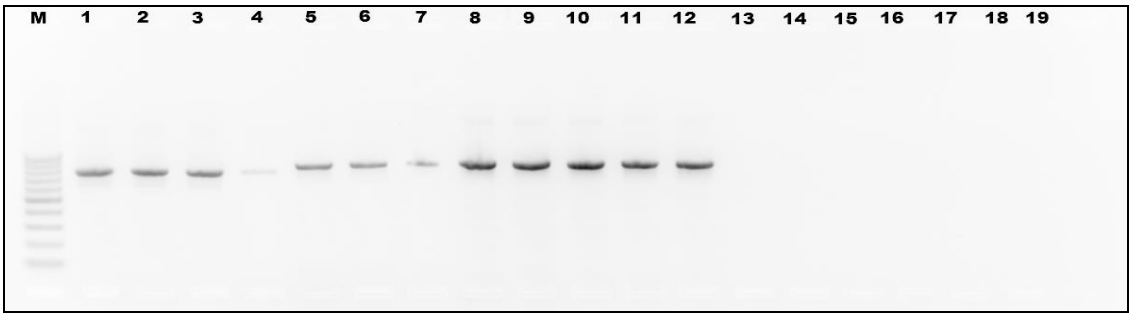

Fig 2. Agarose gel electrophoresis of PCR products i.e. Lanes 1-12 DNA extracted from 12 isolates of *A. flavus*: Lanes 13-19: DNA of seven isolates of *A. niger* isolates using *AflR1-AflR2* primers with 796 bp; Lane M: 100-bp DNA ladder size marker (Jena Bioscience).

production by toxigenic strains (Fente et al., 2001; Ordaz et al., 2003). SMKY was chosen because in a study by Roy et al. (2003), it was shown that it promotes AF production. Molecularly, some selected primers were used in screening experiments applied to all 21 isolates belonging to the *Aspergillus* genera in order to identify potential AF producing isolates. DNA of all the *Aspergillus* isolates as listed in Table 2 were amplified using the primer pair *Anigf* and *Anigr* specific to *A. niger* designed on the basis of the sequence alignment. A single fragment of about 500 bp was only amplified when genomic DNA from *A. niger* isolates was used. No amplification product was observed with genomic DNA from *A. flavus* isolates and the other fungal species tested. The interpretation of the results revealed that PCR assay did not correlate with aflatoxigenicity. The primers i.e. *aflR* and *FLA* amplified positively with the template DNA of *A. flavus* isolates. Both primers gave amplicons of expected size 796 bp and 500 bp with *A. fla*vus isolates. However, the primers could not differentiate AF producers from non-producers. In non-producers, the genes for AF production may be present, but may not be expressed to make functional gene product. Among the two primers, *aflR* was specific to template DNA from *A. flavus* and *A. parasiticus* fungi. The *aflR* gene is known to regulate AF biosynthesis and *Omt* gene is involved in the conversion of sterigmatocystin to o-methylsterigmato-cystin in AF biosynthetic pathway. Lee *et al.* (2006) suggested for safety reasons that *aflR* gene presence could be used to detect AF production by *A. flavus* isolates. Vanden et al. (2001) stated that all *aflR* and *Omt 1* detected isolates of *A. flavus* turned out to be non-producers of AF. Zhou et al. (2000) detected five other commonly found fungal species along with *A. flavus* in indoor environment and found that a minimum of two fungal spores were needed for successful amplification by single primer within a time period of 5–6 hrs. Contamination in the PCR mixtures, interference of related fungi with the target fungus (Klingspor and Loeffler, 2009), shared genes for different mycotoxins (sterigmatocystin and aflatoxin), disrupted gene presence without the production of toxin, etc. are few problems that may provide false results.

Materials and Methods

Feed samples

Twenty samples of fodder including natural feed (14 samples) and compound feed (6 samples) used in camel feeding in Saudi Arabia were collected from various animal feed factories, storehouse and fodder markets from vendor or distributors during 2009. Two sites were selected for the study. The first site was an area south of Riyadh (Wadi-

Table 3. Comparison of aflatoxigenic and non-aflatoxigenic isolates responses after three incubation days at 28 ˚C in media tested.

No.	Identification	Fluorescence detection under UV light (365 nm)					
		ADM	PDA+NaCl	CA	CZ	AFPA	YES
1	*Aspergillus flavus*	+	+	+	-	+	-
2	*Aspergillus flavus*	+	+	+	-	+	-
3	*Aspergillus flavus*	+	+	+	-	+	-
4	*Aspergillus flavus*	+	-	+	-	+	-
5	*Aspergillus flavus*	+	+	+	-	+	-
6	*Aspergillus flavus*	+	+	+	-	+	-
7	*Aspergillus flavus*	+	+	+	-	+	-
8	*Aspergillus flavus*	+	+	+	-	+	-
9	*Aspergillus flavus*	-	+	-	-	-	-
10	*Aspergillus flavus*	-	+	+	-	-	-
11	*Aspergillus flavus*	-	+	+	-	-	-
12	*Aspergillus flavus*	-	+	-	-	-	-
13	*Aspergillus niger*	+	+	+	-	+	-
14	*Aspergillus niger*	+	-	-	-	+	-
15	*Aspergillus niger*	+	-	-	-	+	-
16	*Aspergillus niger*	-	+	-	-	-	-
17	*Aspergillus niger*	-	+	-	-	-	-
18	*Aspergillus niger*	-	+	-	-	-	-
19	*Aspergillus niger*	+	+	+	-	+	-
20	*Aspergillus niger*	+	+	+	-	+	-
21	*Aspergillus niger*	+	+	-	-	+	-

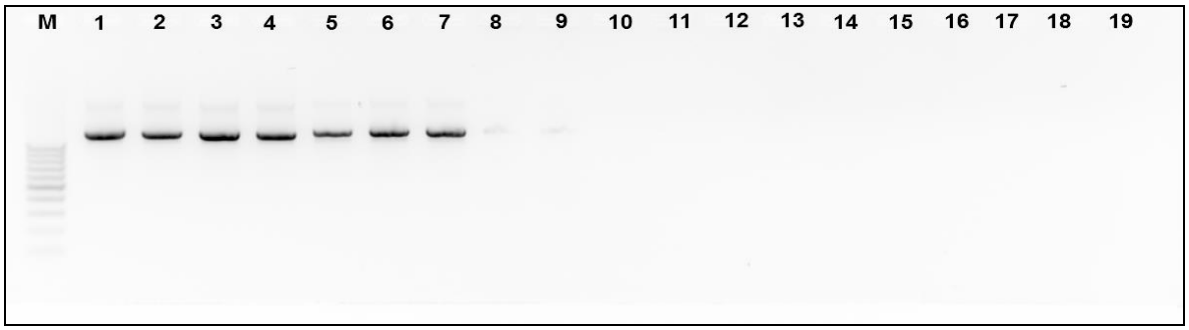

Fig 3. Agarose gel electrophoresis of PCR products i.e. Lanes 1-9: DNA extracted from 7 isolates of *A. niger*; Lanes 10-19: DNA of twelve isolates of *A. flavus* isolates using OMt1R-OMt1F primers with 1232 bp; Lane M: 1232-bp DNA ladder size marker (Jena Bioscience).

Table 4. List of primers used in the current study.

Primer code	Primers Sequence	Annealing	Reference
AnigF	5′-ATTCGCCGGAGACCCCAACA -3′	55	Lezar and Barros, 2010
AnigR	5′-TGTTGAAAGTTTTAACTGATTGCATT-3′		
ASP1P,	5′CAA GCM CGG CTT GTG TGT TGG GTC GYC GTC-3′	60	Suanthie, 2009
ASP2P,	5′AAA GGC AGT GGC GGC ACC ATG TCT GGT-3′		
AflR-1	5′-AACCGCATCCACAATCTCAT-3′	50	Somashekar et al. 2004
AflR-2	5′-AGTGCAGTTCGCTCAGAACA-3′		
FLA1	5´-GTAGGGTTCCTAGCGAGCC-3´	50	González-Salgado et al. 2008
FLA2	5´-GGAAAAAGATTGATTTGCGTTC-3´		
Omt-1F	5′-GGC CCG GTT CCT TGG CTC CTA AGC-3′	65	Hashemi et al. 2007
Omt-1R	5 ′-CGC CCC AGT GAG ACC CTT CCT CG-3′		

Aldawaser Governorate) and the second was east of Riyadh city (Aziziyah area). Fungal species isolates were isolated from different feeds including wheat bran, barley grain, fresh barely, compound feed, crushed corn, sorghum, and millet grain.

Fungal isolation from feeds

Ten pieces from feed samples were used either after being surface-sterilized (using 1% sodium hypochlorite solution and washed three times with sterile distilled water), or without sterilization. Five pieces in each case were placed randomly on the surface of Petri-dishes containing Potato Dextrose agar (PDA) in triplicates. Plated grains were incubated at 25±2 ºC and plates inspected after 3, 5 and 7 days of incubation, using a stereomicroscope (Nikon SMZ-U) to detect fungal growth. After 7 days of incubation, all fungi belonging to genus *Aspergillus* and other genera were transferred into 9 cm Petri dishes containing 15 mL of PDA and examined daily for five days, after which the colonies

developing from the grains were counted. Isolated fungi were purified either by single spore or hyphal tip methods and then transferred to slanted PDA. *Aspergillus* species (Table 2) were identified according to taxonomic schemes proposed by Pitt and Hocking (1997).

Aflatoxigenic ability of the isolates on different culture media

Production of AFs was readily detectable by direct visualization under UV light of a beige ring surrounding colonies after an incubation period of 10 days at 28 °C. When present, the ring exhibited blue fluorescence. Six culture media including PDA+ 20% Na Cl, ADM (25 g tryptone, 20 g yeast extract, 0.5 g ferric citrate and 25 g agar) (Bothast and Fennel, 1974), Coconut agar (CA), Czapek agar (CZ), *Aspergillus flavus* and *parasiticus* Agar (AFPA) (Pitt et al., 1983) and yeast extract sucrose agar (YES) were used to screen for AF production (Hara et al., 1974).All tested isolates were grown on SKMY medium (200 g sucrose, 0.5 g magnesium sulphate, 3 g potassium nitrate, 7 g yeast extract and 1 L of distilled water) for 10 days at 25±2°C (Diener and Davis, 1966). After incubation, the whole flask content was placed in a high speed blender containing 5 grams of sodium chloride and blended for 2-3 min with and then filtered through a glass filter paper. 100 ml of the fungal filtered were centrifuged at 3500 g for 10 min at 4 °C. The upper layer was removed and discarded samples were further diluted 20 times (v/v) with deionized water. The suspension was filtered using a Millipore (0.45 µm in diameter) and filtrate centrifuged at 2700 g for 15 min at 15 °C. The upper layer was removed and the aqueous-methanol layer (100 µl) was added to 0.01 M PBS (900 µl, dilution 1:10). The AF content was analytically determined using 100 µl of this solution.

Detection and quantification of aflatoxins by high performance liquid chromatography (HPLC)

The HPLC system consisted of a Shimadzu liquid chromatography (Shimadzu, Kyoto, Japan) equipped with a Shimadzu SPD-M10A UV Fluorescent detector set at an excitation and emission wavelengths of 362 and 460 nm, respectively, for the detection of AFs. The analytical column was an ODS C18 (4.6x250 mm 5 um in diameter). Standards of aflatoxins (AFs) and maltoryzine were purchased from SIGMA (St. Louis, MO, USA), The AFs standards was used for the calibration and determination of AFs. Methanol-water (60: 40 v/v) was used as the mobile phase pumped at a flow rate 1 mL min^{-1}.

DNA extraction

Fungal mycelium was produced in 20 mL of liquid medium (24 g/L of potato dextrose broth [PDB, Difco Laboratories, Detroit, MI]). Mycelia were harvested by filtration through mesh sieves (40 mm), washed using sterile water and deposited onto a Whatman filter paper to remove excess water. Mycelia were ground to fine powder in a mortar using liquid nitrogen and DNA extracted by the method of Bahkali et al. (2012).

Specific-PCR Assay

Molecular characterizations were applied to 21 isolates of *Aspergillus flavus* and *Aspergillus niger* from Saudi Arabia camel feeds. Specificity was determined using purified DNA from *A. niger* (ASP-KSU1), *A. flavus* (ASP-KSU2), *A. ochraceus* (ASP-KSU8), *A. carbonarious* (ASP-KSU8), *Fusarium semitectum*, *Penicillium citrinum*, **and** *Macrophomina phasolinai* isolates. For PCR reaction, 1 µL of the DNA sample solution was mixed with 24 µL of PCR stock solution containing 2.5 µL of 10× PCR buffer (Jena Bioscience, Germany), 2 µL of 25 mM MgCl2, 0.5 µL of 10 mM dNTP mixture, 1 µL of each primer (20 pmol), 0.2 µL of 5 U/µL Taq polymerase (Jena Bioscience, Germany) and 16.3 µL sterile deionized H_2O. The amplification thermal parameters was: 4 min at 95 °C followed by 40 amplification cycles at 95 °C for 30 sec, (annealing temperatures for each primer sequence are listed in Table 4) for 30 sec and 72 °C for 20 sec. PCRs were performed using a Techne thermal cycler (TC-312-Techne). The purity of the PCR products was determined by gel electrophoresis in a 1.5 % agarose gel in Tris acetate (TAE) buffer. Ethidium bromide (0.5 µg μL^{-1}) stained gels were visualized under UV light and documented in a gel-doc system (Uvitec, Cambridge, United Kingdom). The list of primers and their DNA sequences (Table 4) which were used in the PCR amplification experiments were reported in previous studies (Somashekar et al., 2004; González-Salgado, et al., 2008: Suanthie, 2009; Lezar and Barros, 2010).

Conclusions

Most *A. flavus* isolates were capable of producing detectable levels of both B and G types AF and maltoryzine, although 4 of the 7 *A. niger* isolates failed to produce any quantifiable AF. The presence of toxigenic *A. niger* isolates will increase the risk of feed contamination with AFs and more specifically with AFB1. The genomic PCR and ADM were useful to detect *A. flavus* and *A. niger* isolates, however, they did not distinguish between producers and non-producers of AF. Thus, an extensive survey is needed to further confirm these PCR data in discriminating aflatoxigenic isolates of *A. niger* and *A. flavus* contaminating feed products in Saudi Arabia. Hence, there is a need to develop a simple, rapid method to detect aflatoxigenic fungi, especially to differentiate between producers and non-producers of AF.

Acknowledgments

This project was supported by King Saud University, Deanship of scientific research, College of Science Research Centre.

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