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RESEARCH ARTICLE

PREVALENCE AND DETECTION OF TOXIGENIC *A. FLAVUS, A .NIGER AND A. OCHRACEUS* BY TRADITIONAL AND MOLECULAR BIOLOGY METHODS IN FEEDS

[2]El-Hamaky, A.M., [*,2]Atef A. Hassan, [1]Heidy Abo El Yazeed and [1]Refai, M. K.

[1]Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Egypt.
[2]Department of Mycology and Mycotoxins, Animal Health Research institute,
Agriculture Research Center, Cairo, Egypt

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ABSTRACT

A total of one hundred feed samples were collected from Cairo and Giza governorates and screened for aflatoxigenic and ochratoxigenic fungal isolates. A total of 106 fungal isolates comprising, *Aspergillus flavus, A. ochraceus* and *A. niger* wererecovered from feed samples and tested for aflatoxins and ochratoxin A (OTA) production. The most predominant isolate was *A. flavus,* which was recovered at the range of (40-55%), followed by *A. niger* (30-50 %) and *A. ochraceus* (15-20%). Thirty three of 47 *A. flavus* isolates produced aflatoxin B_1 and B_2 at average levels of (170-750 ppb), while, 22 of 44 tested isolates of *A. niger* produced OTA with average levels of (100-550 ppb), whereas, 12 of 15 *A. ochraceus* isolatesproduced OTA at average levels of (300-700 ppb). Molecular identification of 16 toxigenic fungal isolates (5 *A. flavus*, 6 *A. ochraceus* and *5 A. niger*) was carried out by PCR. The results of PCR of the DNA extracted from these isolates using ITS primer confirmed the identification of *A. flavus, A. ochraceous and A. niger*. The application of real-time PCR (RT-PCR) system directed against the nor-1 gene of the aflatoxins biosynthetic pathway was applied on the DNA extracted from the 5 selected strains of *A. flavus*. The amplification plot of the DNA samples indicated the presence of nor-1 gene in all aflatoxins-producing *A. flavus* isolates and in only one isolate of the negative aflatoxins-producing *A. flavus*. On the other hand, the use of primer set for amplification of omtB gene responsible for AF production amplified 611 bp fragments bands in all aflatoxigenic *A. flavus*, while, no band was detected in all negative aflatoxinogenic isolates. All the sequenced *A. flavus* isolates were confirmed to belong to *A. flavus* species and were 100% similar to the reference isolates of *A. flavus*. The application of PCR assays for detection of ochratoxigenic fungi using OCRA1/OCRA2 primers, amplified a single fragment of about 400 bp, when genomic DNA from ochratoxigenic A. *ochraceus* and non- ochratoxigenic A. *ochraceus* isolates were tested. No product was observed with genomic DNA from *A. niger* isolates. When, PCR assayswas applied using a pair of primers (Aopks1/Aopks2) for specific detection of ochratoxigenic fungi by targeting the metabolic pathway genes Polyketide Synthase (pks) specific to ochratoxin, a single fragment of about 549 bp was produced with all positive ochratoxigenic *A. ochraceus* isolates and 2 ochratoxigenic *A. niger* isolates. No product was observed with genomic DNA from all negative ochratoxigenic isolates of *A. ochraceus* and *A. niger*. Moreover, the combination of biochemical and molecular methods is needed to correctly evaluate the potential toxicological risk in feed caused by these fungi. Conclusion: the application of molecular biology technique was found to be rapid, highly specific, easy to perform and cost effective method to assist creation the programs used for reducing the risk of harmful effects of toxigenic fungi and their toxins to human and other farm animal's health.

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INTRODUCTION

In spite of progressive advances in harvesting, storage and processing technologies, fungal spoilage still has a major economic impact on world food supplies. The most common and destructive food spoilage fungi are belong to the genera of Aspergillus, Penicillium, Eurotium and Fusarium, although other genera are significant in particular foods and feed (Refai and Hassan, 2013). Recently, the Food and Agriculture Organization (FAO, 2011) reported that in areas such as Asia and Africa, 8–18% of cereals commodities, seeds, fruits and vegetables are lost during postharvest handling and storage and about 13.5% of the total value of grain production was lost. The majority of these losses can be attributed to fungal growth and contamination with mycotoxins. Nearly every food or feed

Corresponding author: Atef A. Hassan,
Department of Mycology and Mycotoxins, Animal Health Research institute, Agriculture Research Center, Cairo, Egypt.

commodity can be contaminated by fungal organisms and many of these fungi are capable of producing one or more mycotoxins, which are toxic metabolites of concern to human and animal health. It is estimated that 25 to 50% of the crops harvested worldwide are contaminated with mycotoxins. The percentage is highest in tropical regions, where, up to 80% of the crops are reported to contain significant amounts of mycotoxins (Smith *et al*., 1994; Hassan *et al*., 2012 and Refai and Hassan, 2013). Because of the toxic and carcinogenic potential of mycotoxins, there is an urgent need to develop detection methods that are rapid and highly specific. These mycotoxins have diverse chemical structures and it is not possible to develop one method to detect all relevant mycotoxins, even if some progress was achieved in the simultaneous detection of several mycotoxins (Dall'Asta *et al*., 2003).The highly advanced physico-chemical methods for the analysis of mycotoxins in use have overcome the entire disadvantages of old methods including highly sophisticated clean-up and/or derivatization procedures (Van Quekelberghe *et al*., 2003). In addition, there are much simpler and faster immuno-chemical methods available (Yong and Cousin, 2001). Therefore, the detection of the mycotoxigenic moulds and genes responsible for mycotoxin production could be an alternative for detection of a mycotoxin itself (Abo El Yazeed*et al*., 2011 and Hassan *et al*., 2012). In developing countries, screening methods of agricultural commodities, foods and animal feeds are based on the morphological characterization of the isolated fungi (Liewen and Bullerman, 1999 and Hamilton, 2000). This approach is however, very time-consuming, labor-intensive and requires the expertise of mycologists and above all possesses the inherent possibility of misclassification, since morphological characters could be highly variable depending on the media and culture conditions. Therefore, a 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. So, molecular biology techniques are increasingly used in the identification of fungi and yeasts (Kamiya *et al*., 2005 and Abo El Yazeed *et al*., 2011). Molecular biology techniques (Geisen, 1998) had been introduced as powerful tools for detecting and identifying fungi. The genomes of several toxigenic fungi, including A. flavus, A. niger, F. graminearum and F. verticillioides have been sequenced (Leisová *et al*., 2006 and Sarlin *et al*., 2006). In general, the accuracy of the PCR was found to be 70-85%, where PCR proved to be a rapid diagnostic technique for detection of pan fungal genome directly from clinical specimens (Bagyalakshmi *et al*., 2007 and Hassan *et al*., 2013). The sensitive and specific nested PCR assay as well as the rapid and quantitative Light-Cycler PCR assay might be useful for the diagnosis and monitoring of fungal infections (Bialek *et al*., 2002 and Abo El Yazeed*et al*., 2008 and 2011). Therefore, the present work was undertaken to evaluate both the traditional and molecular biology methods for detection and characterization of the toxigenic Aspergillus isolates recovered from feed.

MATERIALS AND METHODS

Samples

Atotalof100feedsamples (20 of each of poultry feed, yellow corn, white corn, wheat and soya been) was collected for mycological examination. The samples were collected from different farms in Cairo and Giza governorates in which the animals and poultry suffered from disease problems.

Isolation and identification of Aspergillus speciesby traditional method

Ten grams of each sample were transferred aseptically into sterile blender jar, to which 90 ml of 1% peptone water were added and homogenized in a sterile warring blender for 2 minutes and tenfold serial dilutions of the homogenate were prepared (ICMSF, 1978). One milliliter quantities of the previously prepared serial dilutions were inoculated separately into Petri dish plates and mixed with Sabouraud dextrose agar medium. The plates were then left to solidify after mixing, and incubated at 25°C for 3-5 days. The counts of mould colonies were recorded. Individual suspected colonies were selected depending upon their morphological characters. Stock culture were made from each isolate and monitored on Czapek- Dox, malt extract and potato dextrose (PDA) agar slopes for further identification.The identification of mould species was carried out by observation of macroscopic and microscopic characteristics of mould colonies according to ,Pitt and Hocking (2009) and Refai and Hassan (2013).

Production and estimation of aflatoxins by A. flavus on liquid medium (Gabal *et al*., 1994)

The isolates of Aspergillus flavus recovered from feedwere inoculated into flasks containing 50 ml of sterile yeast extract solution 2% and 20% sucrose (YES). Inoculated flasks were incubated at 25ºC for 15 days. At the end of the incubation period, the flask content was filtered to separate the mycelial mat from YES medium. Both were subjected for estimation of aflatoxins and measured qualitatively by TLC (Bauer *et al*., 1983). The positive samples for aflatoxins in TLC method were measured quantitatively by fluorometric method using specific FGisAfla test standards according to the recommended method of AOAC (1990) and Refai and Hassan (2013).

Production and Estimation of Ochratoxin in liquid medium (Davis *et al*., 1969): The strains of *A. ochraceus* and A. niger that were recovered from feeds were screened for ochratoxin A production in liquid medium (YES). The prepared ochratoxin A was measured qualitatively by TLC (Bauer *et al*., 1983) and the positive samples for aflatoxins were measured quantitatively by fluoroumetric method using specific FGis Ochra test standards (AOAC, 1990 and Ozaslan *et al*., 2011).

Molecular identification of mycotoxigenic Aspergillus spp. isolates by using polymerase chain reaction (PCR) technique

Preparation of fungal isolates

Aspergillus spp. isolates that were recovered from feed were subjected for the detection of genes responsible for aflatoxins and ochratoxins production. A total 16 isolates including , Aspergillus flavus (5 isolates), Aspergillus ochraceus (6 isolates) and Aspergillus niger (5 isolates) were cultured on

Sabouraud dextrose broth medium and incubated at 25°C for 2-3 days then stored at -20°C until used for DNA extraction.

Extraction of DNA: Total genomic DNA was isolated from fresh mycelium according to a mini-prep protocol described by Cenis (1992) and Abd-Elsalam *et al*. (2003). In this method potato dextrose broth medium (Hi-media) was inoculated with fungal mycelium and left at room temperature for three days. After centrifugation at 10,000 rpm for 5 min, mycelial mat was pelleted and washed with 500 µl Tris-EDTA buffer. The mat was then homogenized by hand in 300 µl of extraction buffer for 5 min. One hundred and fifty micro liters of 3 M sodium acetate (pH 5.2) was added and the mixture was cooled to 20°C for 10 min., fungal debris was pelleted by centrifugation at 10,000 rpm for 5 min, the supernatant was transferred to a fresh tube, and an equal volume of isopropanol was added. DNA was then pelleted by centrifugation at 10,000 rpm for 10 min. Excess salt was removed by washing with 70% ethanol, and DNA was re-suspended in Tris-EDTA

Amplification of Internal Transcribed Spacer 1 gene of Aspergillus species, Aflatoxin and ochratoxin (Polanco*et al*., 1995 and Paterson, 2006)

To identify the Aspergillus spp. isolates at the molecular level, DNA samples from A. flavus, A. niger and *A. ochraceus* isolates were subjected to PCR analyses using species specific ITS , Afla and Ochra primers. The PCR reaction was carried out in 25µl volume containing 10 ng of DNA sample, 10X Taq polymerase buffer (AB-gene Housse, UK), 25 mM MgCl2, 2 mM dNTPs, 20 pmol of each forward and reverse primer and 0.5 µl (3U/µl) of Red Hot Taq DNA polymerase(ABgene Housse, UK). PCR was performed using AB, (Applied Biosystems) Thermocycler. Samples were heated to 94 ºC for 5 min and then subjected to 35 cycles of 1 min at 94 ºC; 1 min at 58 ºC and 1 min at 72 ºC. The final extension was set at 72°C for 10 min. Ten µl of the PCR product was electrophoresed on 1.5% agarose gel, stained with ethidium bromide, illuminated and documented using Biorad UV Transilluminator.

The sequence of species specific ITS gene for Aspergillus spp., Afla and Ochra primers (Mirhendi *et al*., 2007)

F: 5'-TCCGTAGGTGAACCTGCG G-3' and
R: 5'-TCCTCCGCTTATTGATATGC-3').

The primers sequences for aflatoxin OmtB gene
(Rahimi *et al*., 2008)

OmtB.F: 5'-ATGTGCTTGGGXTGCTGTGG-3',
R: 5'-GGATGTGGTYATGCGATTGAG-3'

The primers sequences for detection of ochratoxin gene
(Reddy *et al*., 2013)

Aopks1: CAGACCATCGACACTGCATGC,
Aopks2: CTGGCGTTCCAGTACCATGAG.

The primers sequences for detection of Polyketide Synthase
OCRA1:CTTCCTTAGGGGTGGCACAGC
(Patiño *et al*., 2005)
OCRA2: GTTGCTTTTCAGCGTCGGCC (Patiño *et al*., 2005).

Sequencing of the DNA of the Amplified Internal Transcribed Spacer gene to identify the species of the isolated Aspergillus species (Mirhendi *et al*., 2007 and Patino *et al*., 2005).

In order to determine the genetic polymorphism between the isolated Aspergillus species, genomic DNA was isolated from three isolates, each of A. flavus, A. niger and *A. ochraceus* and were subjected to RAPD analysis. Five oligonucleotide primers out of twenty-two random 10-mer primers (Operon Technology, Inc., Alameda, CA, USA) tested were used to detect the polymorphism among the different Aspergillus isolates tested. The reaction mixture (20 ml) contained 10 ng DNA, 200 mM dNTPs, 1 M primer, 0.5 units of Red Hot Taq polymerase (AB-gene Housse, UK) and 10-X Taq polymerase buffer (AB-gene Housse, UK). Samples were heated to 94 ºC for 5 min and then subjected to 35 cycles of 1 min at 94 ºC; 1 min at 35C and 1 min at 72 ºC. The amplification products were separated in 1% (w/v) agarose gel in 1 x TBE buffer and visualized by staining with ethidium bromide and photographed with a Polaroid camera. The gel images were scanned using the Gel Doc 2000 Bio-Rad system and analyzed with Quantity One Software v. 4.0.1 (Bio-Rad Laboratories, Hercules, CA USA). The bands were sized and then binary coded by 1 or 0 for their presence or absence in each genotype. The statistic ver. 7 (SPSS, 2006) computer programs were used to calculate the pairwise difference matrices (Yang and Quiros, 1993).

Sequencing reaction (Altschul *et al*., 1990)

A purified PCR product was sequenced in the forward and reverse directions on an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130,USA).Using a ready reaction Bigdye Terminator V3.1 cycle sequencing kit. (Perkin-Elmer/Applied Biosystems, Foster city, CA) cat-number 4336817.A BLAST® analysis (Basic Local Alignment Search Tool) was initially performed to establish sequence identity to GenBank accessions. The sequence reaction was done by addition 2µl of Big dye terminator v.3.1 to 1 ul corresponding primer and 1-10 ul of template according to quality of band and concentration of DNA and complete the total volume to 20 ul by deionized water and finally mix well briefly. The used temperatures ranged from 50-96 oCfor 5 seconds -2 minutes.

Phylogenetic analysis (Mirhendi *et al*., 2007)

A comparative analysis of sequences was performed using the CLUSTAL V multiple sequence alignment program, version 1.83 of MegAlign module of Lasergene DNAStar software (Madison, WI). Pairwise sequence alignments and phylogenetic comparisons of the aligned sequences for the HA and NA genes were also performed with the MegAlign module of Lasergene DNAStar software (Madison ,WI) to determine nucleotide and amino acid sequence similarities and relationships. AIV sequences used for the alignments were obtained from the GenBank and EMBL database using multi sequence alignment and sequences were then presented using BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

Statistical analysis

Data obtained were statistically analyzed for the mean and standard deviation of the mean. Significance of the results was determined by conducting a one way analysis of (F- test) and least significant difference between pair groups as well as t-student test (SPSS 14, 2006).

RESULTS AND DISCUSSION

Fungal pathogens pose serious problems worldwide for human and animal health, especially in the subtropical and tropical regions. Fungi and their toxins are natural contaminants of foods and feeds even when the most efficient condition of culture, harvest, storage and handling are used. The prevalence of moulds in feed samples varies depending on geographical location and season of the year and the specific qualities of climate, vegetation and land are the important factors affecting the prevalence of moulds in connection with a certain geographical location. Mould spores can disperse in the air with the wind or in combination of wind and rain (Mikulec *et al*., 2005). Hassan *et al*. (2011) examined feeds including tibn, hay and processed feeds (20 of each) for fungalpolution. Nine genera of moulds and 2 genera of yeast were recovered from feed samples. The most predominant isolates of all types of feeds were the moulds of genus Aspergillus particularly A. flavus (95%). Other genera as Penicillium sp. (60%), Fusarium sp. (28.3%) and Alternaria sp. (40%) were isolated in variable frequency. The prevalence of fungi in feed was studied by Hassan *et al*. (2012), who reported that the most common isolated moulds from feed were Aspergillus spp. (100%), Fusarium spp. (24%), Mucor spp.(28%), Pencillium spp.(52%), Cladosporium spp.(8%) and Alternaria spp.(28%). In addition, it has been estimated that 25% of the world's crop production is contaminated with mycotoxins.

In the present work, one hundred feed samples (20 of each of poultry feeds, yellow corn, white corn, wheat and soya beans) were subjected for mycological examination. Ninety two samples were contaminated with different species of fungi. All the examined yellow corn and white corn samples were contaminated with fungi (100%), while, in poultry feeds and wheat, the fungi were recovered from 90% of examined samples, whereas in Soya beans samples, 80% of samples were contaminated with moulds. The highest average of total colony count of fungi per gram of the examined samples was in yellow corn(1.46 × 103), followed by poultry feeds (1.43 × 103), white corn (1.37 × 103), wheat (1.37 × 103) , and Soya beans (9.37 × 102) (Table, 1).On the other hand, the highest average of total colony count per gram of the isolated Aspergillus species was in yellow corn (8.95 × 102), followed by poultry feeds (8.73 × 102), wheat (8.15 × 102), white corn (8.05 × 102) and Soya beans (6.37 × 102). While, the Aspergillus species were recovered from 86% of feed samples with the highest incidence in white corn (95%), followed by yellow corn (90%), poultry feed (85%) and wheat and soya bean (80%). These findings are in agreement with the results of Arakawa (2007) and Hassan *et al*. (2008). Regarding the conventional identification of different Aspergillus species, the most predominant isolate was A. flavus which was recovered from poultry feed at the rate of (55%) followed by A. niger (30%) and *A. ochraceus* (15%). In case of yellow corn samples, A. flavus was recovered from (45%), A. niger (50%) and *A. ochraceus* (15%).Whereas, in white corn, A. flavus was yeilded from (50%), A. niger (45%) and *A. ochraceus* (20%) of samples. In wheat samples, A. flavus was recovered from (40%), A. niger (50%) and *A. ochraceus*from (15%). In addition, in Soya beans, A. flavus and A. niger were isolated from (45%) and *A. ochraceus* (10%). In general, the breeding factors such as animal housing, feeding on moldy hay and ventilation system orother environmental factors such as temperature, wind and dew increase the odds of contracting the fungal contamination and infection (Chihaya *et al*., 1991 and Moubasher, 1995).On the other hand, Aspergillus flavus and A.ochraceus were recorded to constitute a public health hazard due to production of aflatoxins and ochratoxins that cause some degree of acute toxicity when consumed in high amounts and are potential carcinogen. In developing countries, it appears that there is a direct correlation between dietary aflatoxins intake and the incidence of liver cancer(FDA, 2000 and Bahtnager *et al*., 2002).The mycotoxins are formed by certain fungal species, whenever environmental factors are conductive during the growth of these frequently occurring mycomycetes on foodstuffs and animal feeds; the process takes place as a secondary metabolism. The mycotoxin inhibits cell division, RNA/ DNA synthesis and causes apoptosis (Rotter *et al*., 1996). Aflatoxins (AFs) are produced mainly by Aspergillus flavus and Aspergillus parasiticus (Bennett and Klich, 2003) and usually found in various agricultural commodities (Meissonnier *et al*., 2008), which are known to be very dangerous mycotoxins. The exposure to aflatoxin B1 can result in suppressed immune response, malnutrition, proliferation of the bile duct, centrilobular necrosis, fatty infiltration of the liver, hepatic lesions and even hepatomas. It is one of the most commonly found metabolites and has a highest toxigenic effects (Richard, 2007). The effects of mycotoxins in human and animals varied from carcinogenic; nephrotoxic and immunosuppressive health effects (Morris *et al*., 1997 and Hassan *et al*., 2009). Although the main route of human exposure to mycotoxins has been identified as the direct ingestion of contaminated cereals, grains and food of animal origin (Morris, *et al*., 1997). There are many studies about whether or not the ingestion of meat, milk, and eggs originating from mycotoxin exposed food production animals is a significant pathway for mycotoxins among humans (Wafia and Hassan, 2000 and Hassan, 2003). However, Samson *et al*. (2004), reported that no strains of A. foetidus produced OTA and the consistent with this analysis, the strain of A. foetidus that was described as an OTA producer was later shown to be A. niger and not A. foetidus. In other study, Hassan *et al*. (2010) detected aflatoxins in 30% of feed samples with the mean value of 3.4 ± 0.1 ppm and ochratoxins in 20% with the mean values of 2.2 ± 0.02 ppm.

Whereas, T-2 toxins and zearalenone were gained from 20% and 16% of samples with the mean levels of 36.0 ± 1.0 and 22 ± 0.3 ppm, respectively, but fuminosin B1 (FB1) toxin was found in 2% of samples at mean levels of 70 ± 0.01 ppm. Currently, from all the examined feeds samples, 47 suspected aflatoxigenic A. flavus species were isolated with the average colony count of (2.12 – 3.53× 102), while, 33 of 47 isolates produced aflatoxin B1and B2 at average levels of (170-750 ppb). On the other hand, 44 suspected ochratoxigenic A. niger species were recovered from feed samples (100%).

Table 1. Prevalence of Aspergillus species and its colony counts in feeds

Feed samples	Total mould count			Total Aspergillus count		
	NO	%	Average TCC	NO	%	Average TAC
Poultry feeds	18	90	1.43 × 103	17	85	8.73 × 102
Yellow corn	20	100	1.46 × 103	18	90	8.95 × 102
White corn	20	100	1.37 × 103	19	95	8.05 × 102
Wheat	18	90	1.21 × 103	16	80	8.15 × 102
Soya beans	16	80	9.37 × 102	16	80	6.37 × 102
Total	92	92	-	86	86	-

Table 2. Prevalence of Aspergillus speciesmembers in feed samples

Isolates	Poultry feed		Yellow corn		White corn		Wheat		Soya beans		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Aspergillus flavus	11	55	9	45	10	50	8	40	9	45	47	47
Aspergillus niger	6	30	10	50	9	45	10	50	9	45	44	44
Aspergillus fumigatus	2	10	3	15	3	15	1	5	2	10	11	11
Aspergillus ochraceus	3	15	3	15	4	20	3	15	2	10	15	15
Aspergillus terreus	2	10	2	10	2	10	3	15	2	10	11	11
Aspergillus parasiticus	1	5	2	10	1	5	1	5	0	0	5	5
Aspergillus glaucus	3	15	2	10	3	15	2	10	3	15	13	13
Aspergillus candidus	3	15	1	5	1	5	2	10	1	5	8	8

Table 3. Prevalence and toxigenicity of isolated Aspergillus flavus, A. niger and *A. ochraceus* from feeds and levels of produced mycotoxins

Aspergillus isolates	Incidence of Aspergillus isolates			Toxigenecity of Aspergillus isolates			Average levels of produced toxins(ppb)	Types of toxins
	No	%	Average of TC	No of tested	No of +ve	%		
A. flavus	47	47	2.12 -3.53 × 102	47	33	70.2	170-750	AFB1 +B2
A. niger	44	44	1.80 -3.10 × 102	44	22	50.0	100-550	OA
A. ochraceus	15	15	0.25-0.85 x 102	15	12	80.0	300-700	OA

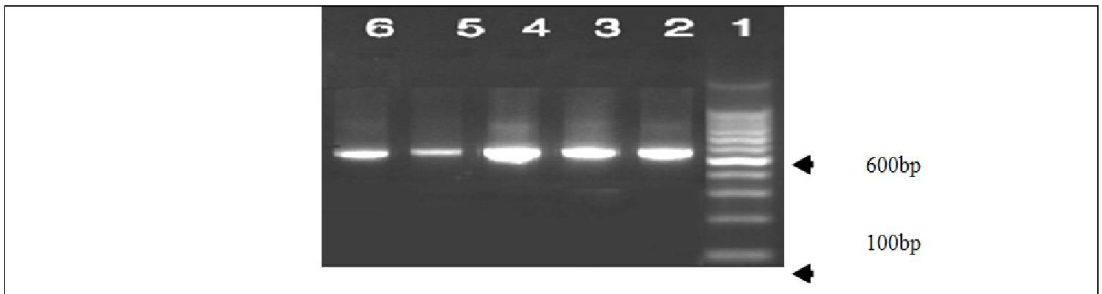

Figure 1. The PCR amplification of the ITS region of Aspergillus spp (1) 100bp ladder, (2-6) *A. flavus* isolates. The amplicon is 595-600bp

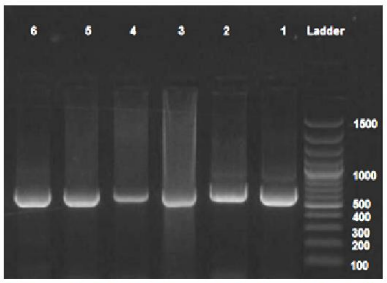

Figure 2. The PCR amplification of the ITS region of Aspergillus spp.(Ladder) 100bp ladder, (1-6) *A. ochraceous* The amplicon is 595-600bp

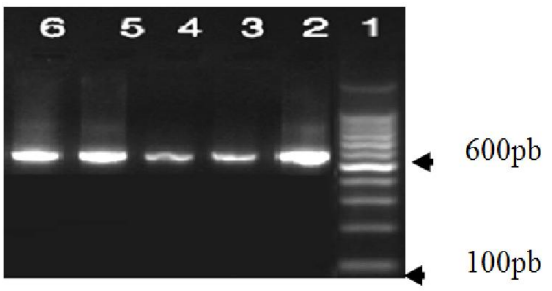

Figure 3. The PCR amplification of the ITS region of Aspergillus spp. (1) 100bp ladder, (2-6) *A. niger* isolates. The amplicon is 595-600bp.

Table 4. Results of using oligonucleotide primers encoding for nor-1 gene for detection of aflatoxinogenic strains by using Real time PCR technique

A. flavus	Mycotoxin profile	Aflatoxin results using nor-1 gene	CT
1	Negative	Negative	-
2	Negative	Positive	32.33
3	Positive	Positive	24.54
4	Positive	Positive	26.64
5	Positive	Positive	27.70

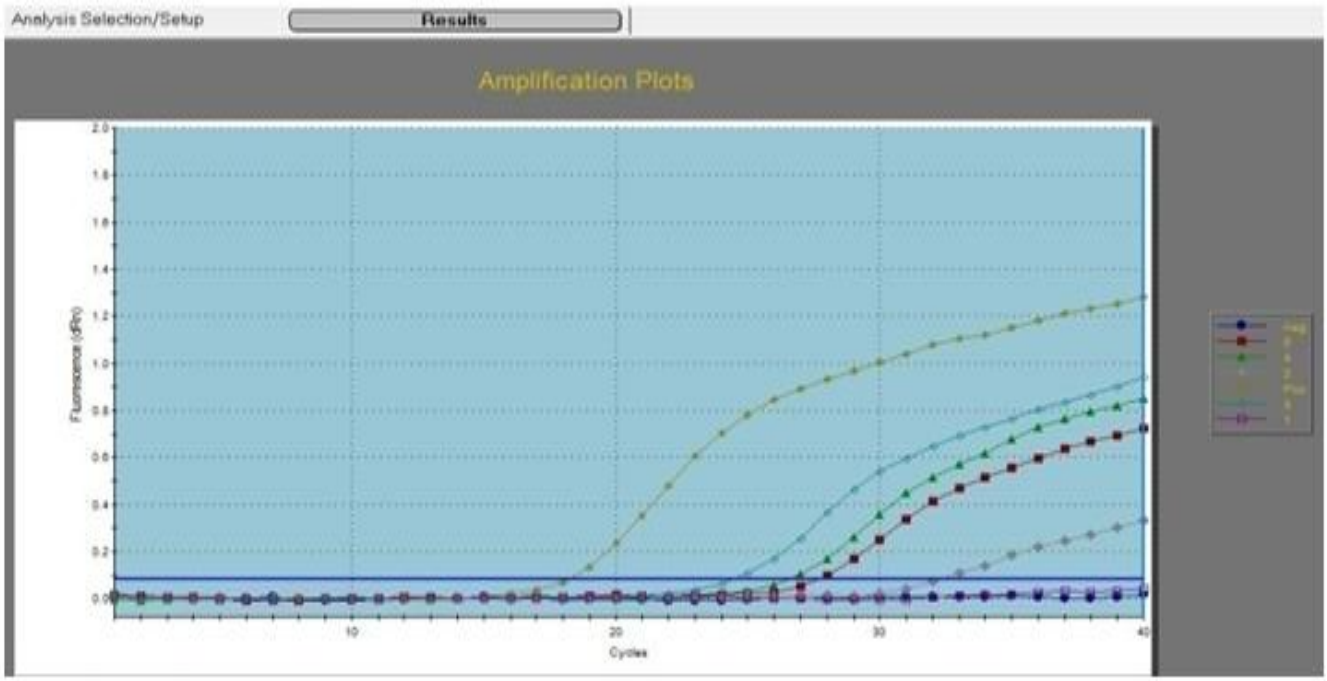

Figure 4. Amplification plots of Real time PCR using oligonucleotide primers encoding for nor-1 gene for detection of aflatoxinogenic A. flavus strains

Table 5. PCR results for detection of omtB gene responsible for production of aflatoxins by A. flavus isolated from feeds.

A. flavus isolates	Mycotoxin profile	PCR Results using omtB.F/omtB.R primers
1	Negative	Negative
2	Negative	Negative
3	Positive	Positive
4	Positive	Positive
5	Positive	Positive

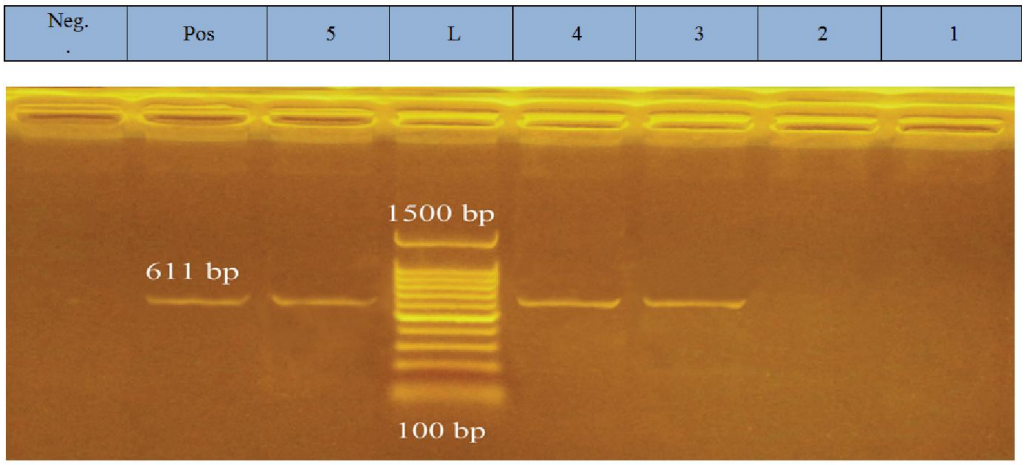

Figure 5. Gel electrophoresis of DNA product of using oligonucleotide primers encoding for omtB gene for detection of aflatoxinogenicity of isolated A. flavus strains by using PCR technique

The average colony count was (1.80- 3.10 × 102), whereas, the average levels of produced OA by 22 of 44 tested isolates of A. niger was (100-550 ppb). In relation to *A. ochraceus* species isolated from the present samples, 15 isolates were recovered with an average colony count of (0.25-0.85 × 102), while, 12 out of 15 tested isolates produced OA at average levels of (300-700 ppb) (Table, 3). The Food and drug administration has established recommended maximum levels for aflatoxins in animal feed as 20 µg/kg of feed (FDA, 1994), while,the permissible limits of aflatoxin for large ruminants varied between 700-1000 ppm of feed. This limit will cause loss of weight gain, high food consumption and low feed efficiency (Keyle *et al*., 1970), whereas, the OA produced significant pathological changes in animal at the levels of 2.5 ppm of animal body weight for 5 days when given orally (Nesheim, 1971).

Table 6. PCR results using OCRA1/OCRA2 primers specific for *A. ochraceus* isolates

Number of Aspergillus isolates	Aspergillus isolates	Mycotoxin profile	PCR results using OCRA1/OCRA2 primers
6	*A. ochraceus*	Positive	Positive
7	*A. ochraceus*	Positive	Positive
8	*A. ochraceus*	Positive	Positive
9	*A. ochraceus*	Negative	Negative
10	*A. ochraceus*	Negative	Positive
11	*A. ochraceus*	Negative	Negative
12	*A. niger*	Positive	Negative
13	*A. niger*	Positive	Negative
14	*A. niger*	Positive	Negative
15	*A. niger*	Negative	Negative
16	*A. niger*	Negative	Negative

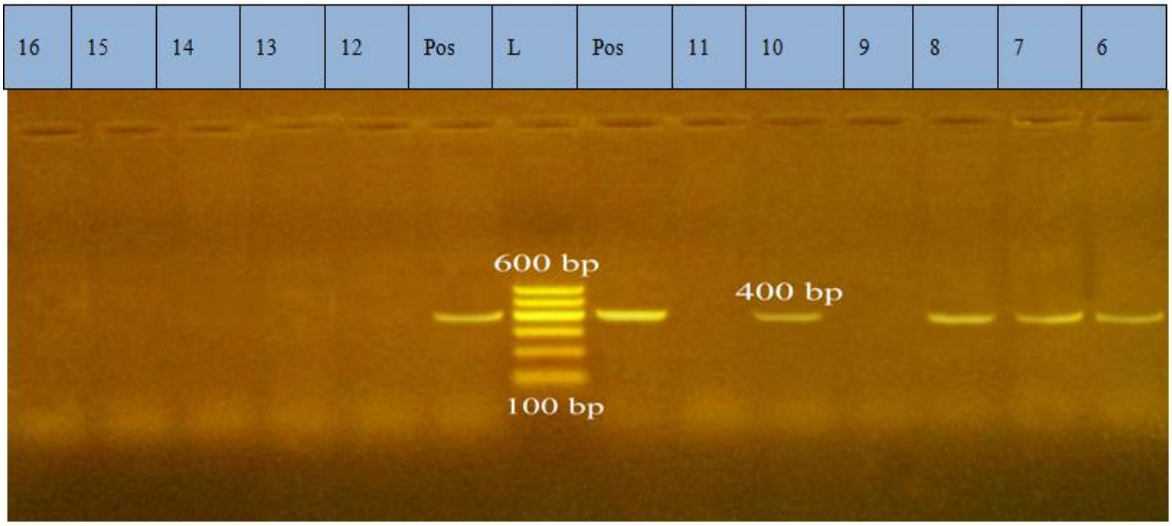

Figure 6. PCR Results using OCRA1/OCRA2 primers which is specific for *A. ochraceus* species

Table 7. PCR Results using Aopks1/ Aopks2primers targeting the metabolic pathway gene (pks) specific to ochratoxin chemotype

Number of Aspergillus isolates	Aspergillus isolates	Mycotoxin profile	PCR results using Aopks1/ Aopks2 primers
6	*A. ochraceus*	Positive	Positive
7	*A. ochraceus*	Positive	Positive
8	*A. ochraceus*	Positive	Positive
9	A. ochraceus	Negative	Negative
10	A. ochraceus	Negative	Negative
11	A. ochraceus	Negative	Negative
12	*A. niger*	Positive	Positive
13	*A. niger*	Positive	Negative
14	*A. niger*	Positive	Positive
15	*A. niger*	Negative	Negative
16	*A. niger*	Negative	Negative
16	*A. niger*	Negative	Negative

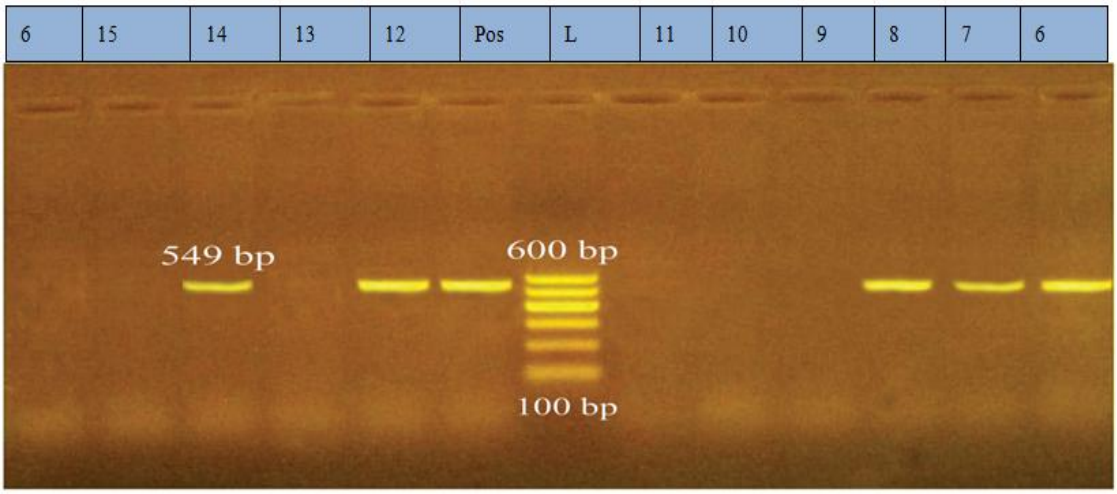

Figure 7. Gel electrophoresis of DNA product of using Oligonucleotide primers encoding for (pks) gene

The detected levels of mycotoxins in the present study were significantly over the permissible limits in feeds as a result of continuous feeding of toxiocated feed. The same findings were detected by Hassan *et al*. (2009, 2010, 2012 and 2014), in association with significant high mycotoxin levels in feed and sera of diseased animals. The pollution of animal feeds results in significant losses in animal health and causes important burdens to the country's economy with regard to meat, milk, wool and leather industries. Therefore, frequent testing program of the animal feeds and their environment for fungi and mycotoxin contamination is a critical demand. The traditional schemes for the isolation and identification of fungi from food and feed samples are time-consuming and require a high knowledge of fungal taxonomy. Even with taxonomic expertise, identification is commonly difficult in some genera of fungi that contain a large number of closely related species. The application of molecular biology techniques can help to overcome these problems because they can reduce the time of identification from days to hours and also allow precise species identification. Polymerase chain reaction (PCR) is a technique that was developed in 1985 for the in-vitro amplification of specific segments of DNA (Saiki *et al*. 1985; Mullis and Faloona 1987). This technique has allowed the precise identification and fast detection of fungal species without the need for isolating pure cultures. The molecular techniques had been introduced as powerful tools for detecting and identifying such mycotoxigenic fungi. The sensitive and specific nested PCR assay as well as the rapid and quantitative Light Cycler PCR assay might be useful for the diagnosis and monitoring of fungal infections (Bialek *et al*., 2002 and Abo El Yazeed *et al*., 2008 and 2011).Molecular methods such as polymerase chain reaction (PCR) have been described to resolve genetic variation among isolates within or between species of fungi (Chandra *et al*., 2008, Chandra *et al*., 2010). Especially, random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphisms (RFLPs) of rDNA have been widely used for assessing genetic diversity, genome mapping, and molecular diagnostics of many fungal species (Annamalai *et al*., 1995 and Sampietro *et al*., 2010). Another strategy to detect genetic variation is the use of defined PCR amplified fragments as substrate for RFLP or sequence analysis. The ribosomal DNA (rDNA) regions had often been chosen for taxonomic and phylogenetic studies, because sequence data are available and contain both variable and conserved regions; despite the discrimination at the genus, species, or intra-specific level. The rDNA repeat includes both highly conserved genes and more variable spacer regions (Abo El Yazeed *et al*., 2011). Baird *et al*. (2008) used a different methodology based on DNA fingerprinting with two consecutive amplifications with arbitrary primers, with which the majority, but not all, of the aflatoxigenic isolates was differentiated from the non-aflatoxigenic.In the present study, 16 fungal isolates recovered from feeds and morphologically and microscopically identified as A. flavus (5 isolates), *A. ochraceus* (6 isolates) and A. niger (5 isolates) were selected from the isolated mycotoxigenic Aspergillus species for molecular identification. The results of PCR for DNA extracted from these isolates using ITS primer as a universal primer confirmed the tested isolates as being Aspergillus spp. (A. flavus, *A. ochraceus* and A. niger). They amplified a 595-600 bp fragment of ITS region of the Aspergillus spp. (Figures 1,2,3). Schmidt-Heydt and Geisen (2007) developed a microarray (DNA chip) that contains oligonucleotides homologous to genes from several fungal species that are responsible for the biosynthesis of mycotoxins. Consequently, this microarray covers most of the known relevant mycotoxin biosynthesis genes. The selection of target sequence specific for a given mycotoxin-producing fungus is a key process in the development of a PCR-based diagnostic assay. These target sequences used for designing PCR primers may be divided into two groups: (a) anonymous DNA sequences and (b) functional genes (Carter and Vetrie, 2004).Anonymous DNA sequences are obtained from an unbiased sample of genomic DNA and may or may not contain functional genes. Developing markers from anonymous sequences requires comparative analyses between the DNA profiles of related species generated from randomly amplified fragments by random amplified polymorphic DNA (RAPD) or amplified fragment length polymorphism (AFLP) (Williams *et al*., 1990 and Vos *et al*., 1995).Both methodologies have proven to be powerful taxonomic instruments, especially at low taxonomic positions. However, Geisen (1996) and Scherm *et al*. (2005) used multiplex PCR with three sets of primers specific for three structural genes of the AF biosynthetic pathway aflD, aflM and aflO and was able to differentiate aflatoxigenic A. flavus and A. parasiticus from other food borne fungi. While, Färber *et al*. (1997) detected aflatoxigenic strains of A. flavus in contaminated figs by performing a monomeric PCR with the same sets of primer used by Geisen (1996).

On the otherhand, multiplex PCR with the AF pathway genes aflR, aflD, aflM and aflO did not produce a clear pattern that would allow to accurately differentiate aflatoxigenic from non-aflatoxigenic strains (Criseo *et al*., 2001). Similarly, Lee *et al*., (2006) detected the differences in the aflR gene of A. flavus, A. oryzae, A. parasiticus and A. sojae, but they were not able to clearly differentiate the species. Such systems had been applied to monitor aflatoxin production and aflatoxin gene expression based on various regulatory and structural aflatoxin pathway genes in A. parasiticus and/or A. flavus (Scherm *et al*., 2005 and Degola *et al*., 2007), and were found to be very rapid and sensitive. So, the genetic protocols that can fully differentiate between aflatoxin producers and non-producers had not yet been successfully established. Furthermore, one had to be aware that some genes are not exclusive of the aflatoxin biosynthetic pathway, which could create false-positives from sterigmatocystin producing fungi (Paterson, 2006).In the present study, a real-time PCR (RT-PCR) system directed against the nor-1 gene of the aflatoxin biosynthetic pathway as target sequence was applied on the DNA extracted from the 5 selected strains of A. flavus. The results of traditional screening method of aflatoxigenicity revealed that, A. flavus isolates No. 1 and 2 were negative producers of aflatoxins, while isolates No. 3, 4 and 5 were positive producer. The amplification plot of the DNA samples indicated the presence of nor-1 gene in all aflatoxins-producing isolates (3, 4, 5) and only one isolate of the negative aflatoxins-producing A. flavus (isolate 2). According to these results the primer/probe system used (nor taq-1, nor taq-2, nor probe) appeared sensitive and accurate for detection of the nor-1 gene fragments extracted from A. flavus isolates. The cycle threshold (CT) values of the isolated A. flavus isolates were higher than that of the positive control

isolate, which indicated the lower number of the DNA copies of the gene in the isolates than that of the control one.On the other hand, the primer set used in this study for amplification of omtB gene that is responsible for aflatoxin production was able to amplify 611 bp fragments bands in all the previously detected positive aflatoxigenic A. flavus isolates by traditional method of screening for AF production (Tables 3, 4, 5), while, no band was detected in all negative aflatoxinogenic isolates (1, 2) as shown in (Table, 4) and (Figure, 4). Currently, a purified PCR DNA product of the Amplified Internal Transcribed Spacer gene using (ITS1 and ITS4) primers of A. flavus isolates no. 2 (negative aflatoxigenic) and no. 3 and 4 (positive aflatoxigenic) was sequenced in the forward and reverse directions on an Applied Biosystems 3130 automated DNA Sequencer and sequence identity. All the sequenced samples belonged to A. flavus isolates were 100% similar to the reference isolates of A. flavus.On the other hand, Schmidt *et al*.(2003) investigated the genetic relatedness among 70 strains of *A. ochraceus* with AFLP markers and detected a number of bands characteristic for *A. ochraceus*. Three of these bands were cloned and sequenced, after which the sequences were used to design three primer pairs specific for *A. ochraceus*. However, the primer pair is able to amplify DNA sequence from both *A. ochraceus* and A. westerdijkiae because it was developed previous to the division of formal *A. ochraceus* species into the two species mentioned above. Other studies by Patiño *et al*. (2005) and Morello*et al*.(2007) developed a specific PCR assay (OCRA1/OCRA2) for the detection of *A. ochraceus* on the basis of ITS sequence comparison between several strains of Aspergillus species. The specificity of the primer pair was tested on a number of Aspergillus sp., Penicillium sp., Cladosporium sp., Botrytis sp., and Alternariasp. strains commonly associated with grapes, cereals, and coffee. A single fragment of about 400 bp was only amplified from the genomic DNA of *A. ochraceus* strains. No product was amplified from genomic DNA from Aspergillus isolates other than *A. ochraceus* or from other genera. According to the authors, the sensitivity of the PCR assay based on ITS sequences was higher (1 and 10 pg of DNA template per reaction) than one based on a single copy gene (0.1 and 1 ng of DNA template per reaction). The authors did not mention the new species A. westerdijkiae, and the primer pair presumably does not distinguish between A. westerdijkiae and *A. ochraceus*.

Furthermore, Samson *et al*. (2007) illustrated that the differences between some species belonging to section Nigri were very slight and their discrimination requires molecular analysis. While, Parenicova´ *et al*. (2001) detected that A. niger sensu stricto, A. tubingensis, A. foetidus and A. brasiliensis are morphologically identical and collectively have been called the A. niger aggregate. The PCR-restriction fragment length polymorphism (RFLP) analysis of the ITS1- 5.8S-ITS2 region allows the four A. niger aggregate taxa to be classified into two patterns (N and T). A. foetidus and A. tubingensis are classified as type T, and A. niger and A. brasiliensis are classified as type N by the Caban˜es group (Accensi *et al*., 1999; Accensi *et al*., 2001). While, Perrone *et al*. (2004) identified regions suitable to design specific PCR primers for the detection of A. carbonarius. The primer pair CARBO1/2 produced a PCR product of 371 bp with a sensitivity of about 12 pg when using pure total genomic DNA. Although the PCR assay was useful in screening isolates of black aspergilli from grapes.Similarly, based on RAPD markers, Sartori *et al*. (2006) developed specific primers to detect A. niger. The primer pair denoted OPX7372F/ OPX7372R generated an amplicon of 372 bp in all A. niger stricto sensu isolates, and no amplification product was observed in reactions using DNA from related species. This PCR assay was successfully applied in detecting A. niger in coffee beans. With the same objective, to quantify A. carbonarius in grape samples, Atoui *et al*. (2006)used a specific primer pair (Ac12RL_OTAF/Ac12RL_OTAR) that was designed from the acyl-transferase (AT) domain of the polyketide synthase sequence (Ac12RL3) to amplify a 141-bp PCR product. Using real-time PCR conjugated with SYBR Green I dye, the authors found a positive correlation (r2 = 0.81) between A. carbonarius DNA content and OTA concentration in 72 grape samples. While, Geisen *et al*. (2004) found a strong correlation between the copy numbers of the otapksPN gene and colony count of A. carbonarius and ochratoxin production. Based on these points, the measurement of mycotoxin gene expression would allow more meaningful monitoring of OTA in food; these genes are frequently expressed some days prior to the mycotoxin production and thus would allow an early warning (Schmidt-Heydt and Geisen 2007).

In the present study, PCR assays were carried out using OCRA1/OCRA2 primers which are specific for *A. ochraceus* species. A single fragment of about 400 bp was only amplified when genomic DNA from *A. ochraceus* isolates was used (all positive ochratoxigenic *A. ochraceus* (isolates no. 6, 7, and 8) and only one negative ochratoxigenic *A. ochraceus* (isolate no. 10). No product was observed with genomic DNA from A. niger isolates as shown in Table (6) and Figure (6).

Currently, PCR assays were carried out using a pair of primers (Aopks1/Aopks2), which were designed and used for specific detection of ochratoxigenic fungi by targeting the metabolic pathway genes Polyketide Synthase (pks) specific to toxin chemo type. A single fragment of about 549 bp was produced with all positive ochratoxigenic *A. ochraceus* (isolates no. 6, 7, and 8) and 2 ochratoxigenic A. niger isolates(isolates no. 12 and 14). No product was observed with genomic DNA from all negative ochratoxigenic isolates of *A. ochraceus* and A. niger as shown in Table (7) and Figure (7).All the sequenced *A. ochraceus* isolates were proved to belong to *A. ochraceus* species and they were almost (98.2- 100%) similar to the reference isolates .While, all the sequenced A. niger isolates were proved to belong to A. niger species and they were (100%) similar to the reference isolates and the sequence of isolate no. 15 was almost (99.8%) identicalto reference isolates. From the foregoing results, it is clear that the present study demonstrates the feasibility of ITS sequencing for the identification of clinically important toxigenic fungi which is highly specific (Leaw *et al*., 2006). Therefore, sequencing rDNA and ITS 2 region is a good supplement for clinical conventional technique in the identification of microbes than traditional methods.

Conclusion

The high incidence of toxigenic fungi in feed and the significant levels of mycotoxins production recorded in

present study warrants that there is urgent need to undertake mycotoxin awareness creation programs among different foods and feedstuffs in Egypt. A combination of biochemical and molecular methods is needed to correctly evaluate the potential toxicological risk in foods and feedstuffs caused by these fungi. Application of molecular biology technique was found to be rapid, highly specific, easy to perform and cost effective method to assist creation of such programs and reduction of the risk of harmful effects of toxigenic fungi and their toxins to human and other farm animals health.

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