



RESEARCH ARTICLE

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

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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* were recovered 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 B₁ and B₂ 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* isolates produced 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. ochraceus* 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 aflatoxigenic 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 assays was 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

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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

A total of 100 feed samples (20 of each of poultry feed, yellow corn, white corn, wheat and soya bean) 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* species by 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 feed were inoculated into flasks containing 50 ml of sterile yeast extract solution 2% and 20% sucrose (YES). Inoculated flasks were incubated at 25°C 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 fluorometric method using specific FGisOchra 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 (AB-gene Housse, UK). PCR was performed using AB, (Applied Biosystems) Thermocycler. Samples were heated to 94 °C for 5 min and then subjected to 35 cycles of 1 min at 94 °C; 1 min at 58 °C and 1 min at 72 °C. 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 °C for 5 min and then subjected to 35 cycles of 1 min at 94 °C; 1 min at 35C and 1 min at 72 °C. 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 tbn, hay and processed feeds (20 of each) for fungal pollution. 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%), *Penicillium* 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 (1.46×10^3), followed by poultry feeds (1.43×10^3), white corn (1.37×10^3), wheat (1.37×10^3), and Soya beans (9.37×10^2) (Table, 1). On the other hand, the highest average of total colony count per gram of the isolated *Aspergillus* species was in yellow corn (8.95×10^2), followed by poultry feeds (8.73×10^2), wheat (8.15×10^2), white corn (8.05×10^2) and Soya beans (6.37×10^2). 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 yielded 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 or other 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 conducive 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 fumonisin 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 $(2.12 - 3.53 \times 10^2)$, while, 33 of 47 isolates produced aflatoxin B1 and 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 × 10 ³	17	85	8.73 × 10 ²
Yellow corn	20	100	1.46 × 10 ³	18	90	8.95 × 10 ²
White corn	20	100	1.37 × 10 ³	19	95	8.05 × 10 ²
Wheat	18	90	1.21 × 10 ³	16	80	8.15 × 10 ²
Soya beans	16	80	9.37 × 10 ²	16	80	6.37 × 10 ²
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.	%
<i>Aspergillus flavus</i>	11	55	9	45	10	50	8	40	9	45	47	47
<i>Aspergillus niger</i>	6	30	10	50	9	45	10	50	9	45	44	44
<i>Aspergillus fumigatus</i>	2	10	3	15	3	15	1	5	2	10	11	11
<i>Aspergillus ochraceus</i>	3	15	3	15	4	20	3	15	2	10	15	15
<i>Aspergillus terreus</i>	2	10	2	10	2	10	3	15	2	10	11	11
<i>Aspergillus parasiticus</i>	1	5	2	10	1	5	1	5	0	0	5	5
<i>Aspergillus glaucus</i>	3	15	2	10	3	15	2	10	3	15	13	13
<i>Aspergillus candidus</i>	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			Toxigenicity of Aspergillus isolates			Average levels of produced toxins(ppb)	Types of toxins
	No	%	Average of TC	No of tested	No of +ve	%		
<i>A. flavus</i>	47	47	2.12 -3.53 × 10 ²	47	33	70.2	170-750	AFB1 +B2
<i>A. niger</i>	44	44	1.80 -3.10 × 10 ²	44	22	50.0	100-550	OA
<i>A. ochraceus</i>	15	15	0.25-0.85 x 10 ²	15	12	80.0	300-700	OA

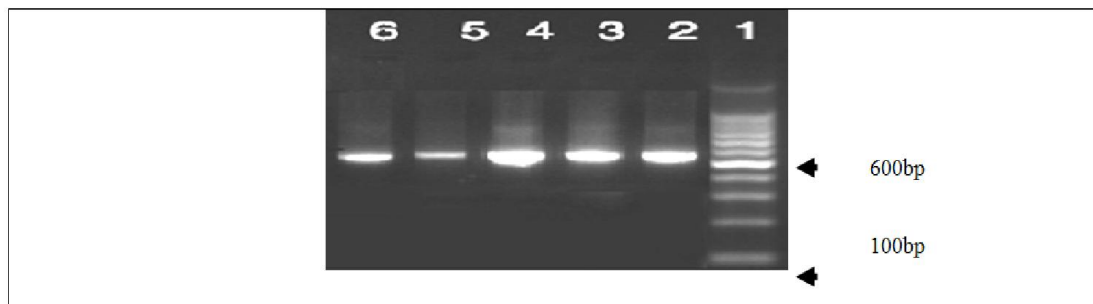


Figure 1. The PCR amplification of the ITS region of *Aspergillus* spp (1) 100bp ladder, (2-6) *A. flavus* isolates. The amplicon is 595-600bp

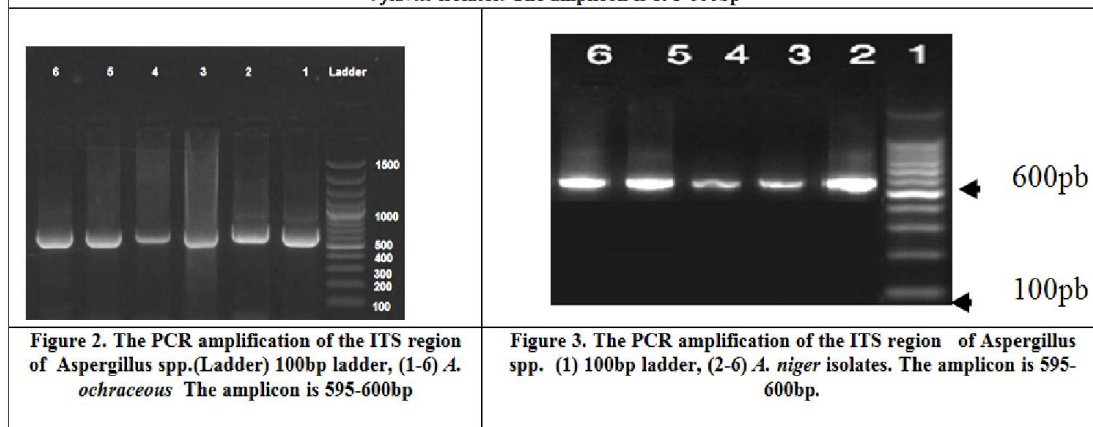


Figure 2. The PCR amplification of the ITS region of *Aspergillus* spp.(Ladder) 100bp ladder, (1-6) *A. ochraceus* The amplicon is 595-600bp

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

<i>A. flavus</i>	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

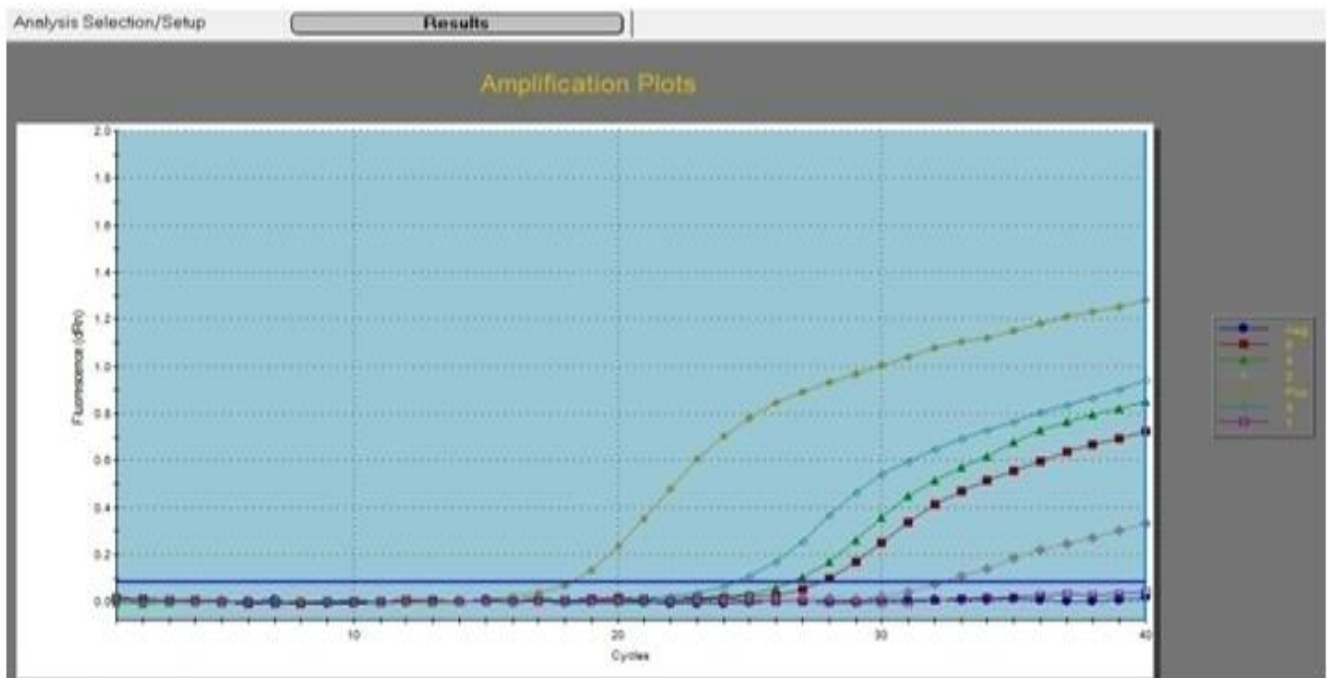


Figure 4. Amplification plots of Real time PCR using oligonucleotide primers encoding for nor-1 gene for detection of aflatoxinogenic *A. flavus* strains

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

<i>A. flavus</i> 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

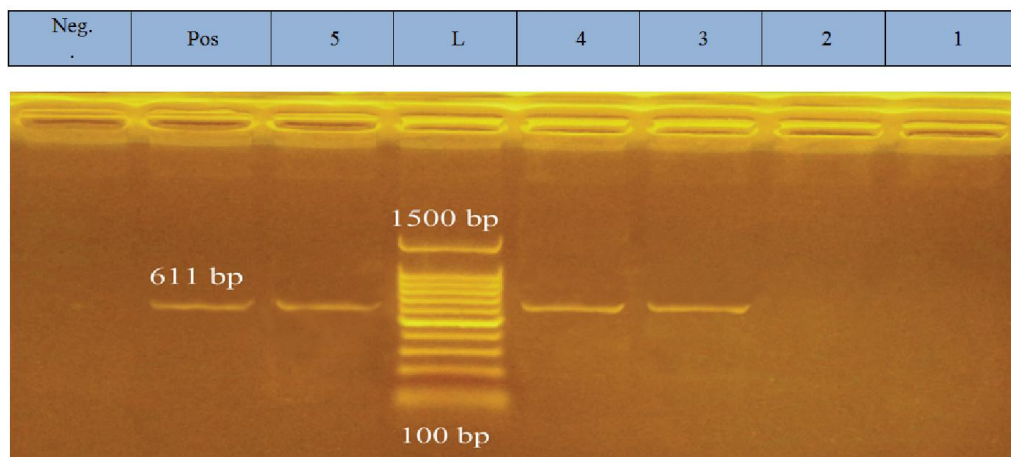


Figure 5. Gel electrophoresis of DNA product of using oligonucleotide primers encoding for omtB gene for detection of aflatoxinogenicity of isolated *A. flavus* strains by using PCR technique

The average colony count was $(1.80-3.10 \times 10^2)$, 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 $(0.25-0.85 \times 10^2)$, 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 $20 \mu\text{g}/\text{kg}$ 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	<i>A. ochraceus</i>	Positive	Positive
7	<i>A. ochraceus</i>	Positive	Positive
8	<i>A. ochraceus</i>	Positive	Positive
9	<i>A. ochraceus</i>	Negative	Negative
10	<i>A. ochraceus</i>	Negative	Positive
11	<i>A. ochraceus</i>	Negative	Negative
12	<i>A. niger</i>	Positive	Negative
13	<i>A. niger</i>	Positive	Negative
14	<i>A. niger</i>	Positive	Negative
15	<i>A. niger</i>	Negative	Negative
16	<i>A. niger</i>	Negative	Negative

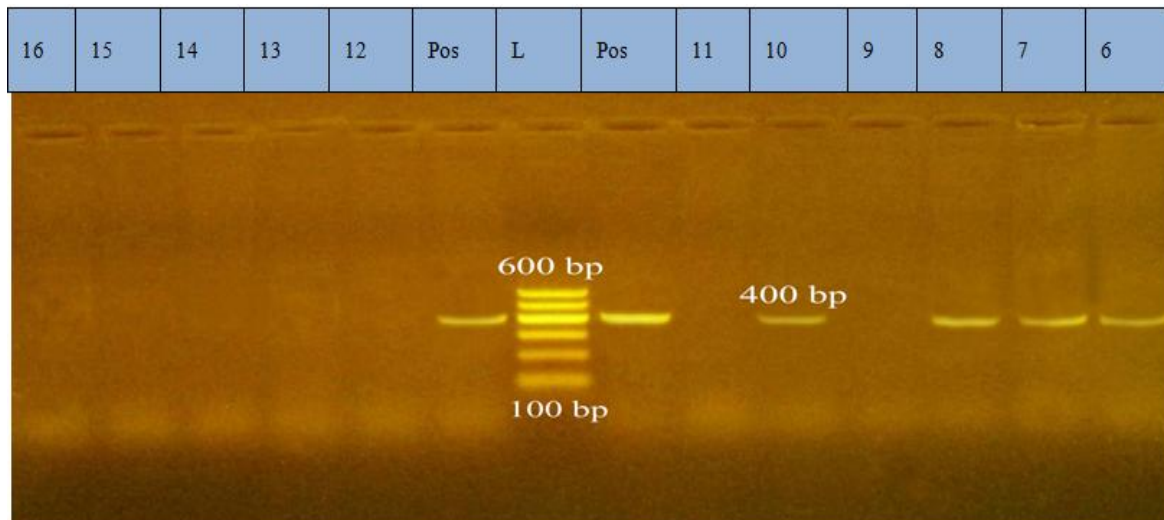
Figure 6. PCR Results using OCRA1/OCRA2 primers which is specific for *A. ochraceus* species

Table 7. PCR Results using Aopks1/ Aopks2 primers 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	<i>A. ochraceus</i>	Positive	Positive
7	<i>A. ochraceus</i>	Positive	Positive
8	<i>A. ochraceus</i>	Positive	Positive
9	<i>A. ochraceus</i>	Negative	Negative
10	<i>A. ochraceus</i>	Negative	Negative
11	<i>A. ochraceus</i>	Negative	Negative
12	<i>A. niger</i>	Positive	Positive
13	<i>A. niger</i>	Positive	Negative
14	<i>A. niger</i>	Positive	Positive
15	<i>A. niger</i>	Negative	Negative
16	<i>A. niger</i>	Negative	Negative
16	<i>A. niger</i>	Negative	Negative

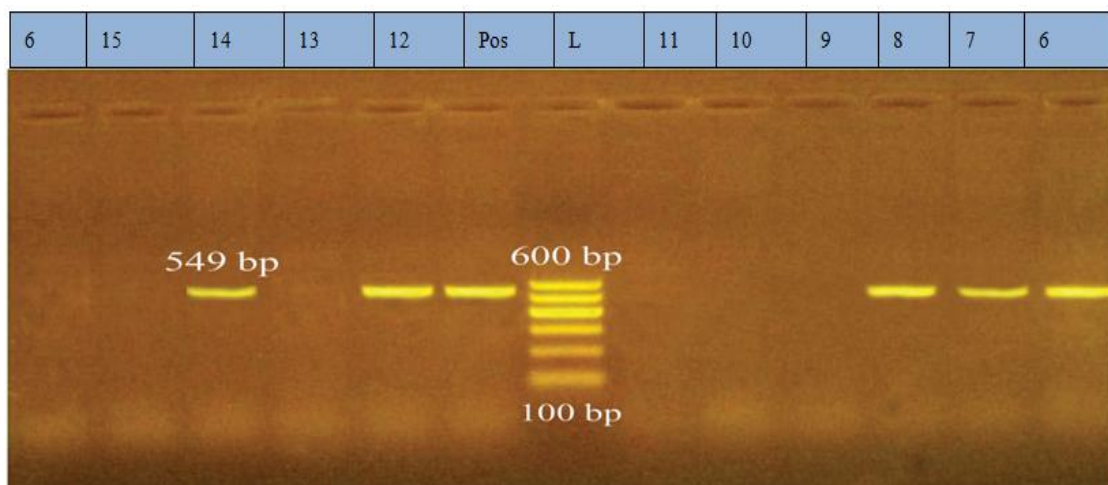


Figure 7. Gel electrophoresis of DNA product of using Oligonucleotide primers encoding for (pks) gene

The detected levels of mycotoxins in the present study were significantly over the permissible limits in feeds as a result of continuous feeding of toxicated 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 Vetric, 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 aflatoxigenic 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 *Alternaria* sp. 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 Cabanes 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 ($r^2 = 0.81$) 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%) identical to 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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REFERENCES

- Abd-Elsalam, K.A., Aly, I.N., Abdel-Satar, M.A., Khalil, M.S. and Verreet, J.A. 2003. PCR identification of *Fusarium* genus based on nuclear ribosomal-DNA sequence data. *African Journal of Biotechnology*, 2 (4): pp. 82-85.
- Abo El Yazeed Heidy; Atef Hassan; Reda E.A. Moghaieb, Mai Hamed and Mohamed Refai, 2011. Molecular detection of fumonisin producing *Fusarium* species in animal feeds using polymerase chain reaction (P.C.R.). *Journal of Applied Sciences Research*, 7(4): 420-427, 2011. ISSN 1819-544X.
- Abo El Yazeid; W.Tawakkol; A.A.Hassan and Eman, Radwan. 2008. Characterization of *Candida albicans* strains by Biochemical and Molecular Biology Techniques. *Bull. Fac. Of Pharm., Cairo Univ., Vol. 46, No. 1 (2008) (Special Issue)*.
- Accensi, E., Abarca, M.L. and Cabanes, F.J. 2004. Occurrence of *Aspergillus* species in mixed feeds and component raw materials and their ability to produce ochratoxin A. *Food Microb.*, 21: 623-627.
- Accensi, F., Caño, J., Figueira, L., Abarca, M.L. and Cabañes, F.J. 1999. New PCR method to differentiate species in the *Aspergillus niger* aggregate. *FEMS Microbiol Lett* 180:191-196.
- Accensi, F., M. L. Abarca, J. Cano, L. Figuera, and F. J. Cabanes. 2001. Distribution of ochratoxin producing strains in the *A. niger* aggregate. *Antonie*
- Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. 1990. *J. Mol. Biol.*, 215, 403-410.
- Annamalai, P., H. Ishii, D. Lalithakumari and R. Revathi. 1995. Polymerase chain reaction and its applications in fungal disease diagnosis. *J. Plant Dis. Protect.*, 102: 91-104.
- Arakawa, K. 2007. Studies on infectious and toxic metabolite activities of saprophytic fun-gi in domestic animal breeding environment. *Bulletin of Nippon Veterinary and Life Science University*, 56: 75-77.
- Association Official Analytical Chemists AOAC. 1990. *Official Methods of Analysis*. 15th Ed., Assoc. of official Analytical chemists, Washington, D. C.
- Atoui A., Dao P., Mathieu F. and Lebrihi A. 2006. Amplification and diversity analysis of ketosynthase domains of putative polyketide synthase genes in *Aspergillus ochraceus* and *Aspergillus carbonarius* producers of ochratoxin A. *Mol Nutr Food Res.*, 50:448-493.
- Bagyalakshmi, R.; Therese, K.L. and Madhavan, H.N. 2007. Application of semi-nested polymerase chain reaction targeting internal transcribed spacer region for rapid detection of panfungal genome directly from ocular specimens. *Indian J Ophthalmol.*, 55 (4): 261-5.
- Bahtnager, D., Yu, J. and Ehrlich, K. 2002. Toxins of filamentous fungi. In: Breitenbach, M., Cramer, R., Lehrer, S. (Eds.). *Fungal Allergy and Pathogenicity Chem. Immunology*, 81. Karger, Basel, pp. 167-206.
- Baird, R., H.K. Abbas, G. Windham, P. Williams, S. Baird, P. Ma, R. Kelley, L. Hawkins and M. Scruggs, 2008. Identification of select fumonisin forming *Fusarium* species using PCR applications of the polyketide synthase gene and its relationship to fumonisin production in vitro. *Int. J. Mol. Sci.*, 9: 554-570.
- Bauer, J., Montgelas, A.V. and Gedek, B. 1983. Aflatoxin B1 production in presence of preservatives antimicrobial agents. *Proc. Int. symp. Mycotoxins, Cairo, Egypt.*, PP. 249-255.
- Bennett, J.W. and Klich M. 2003. Mycotoxins. *Clinical Microbiology Reviews*, 16: 497-516.
- Bialek, R., Weiss, M., Bekure-Nemariam, K., Najvar, L.K., Alberdi, M.B., Graybill, J. R. and Reischl, U. 2002. Detection of *C. neoformans* DNA in tissue samples by nested and real-time PCR assays. *Clin. Diagn. Lab. Immunol.* 9 (2): 461-9.
- Carter, P.N. and Vetrie, D. 2004. Applications of genomic microarrays to explore human chromosome structure and function. *Hum. Mol. Genet.*, 13:297-302.
- Cenis, J.L. 1992. Rapid extraction of fungal DNA for PCR amplification. *Nucl. Acids Res.*, 20: 2380.
- Chandra, S.N., A.C.U. Shankar, S.R. Niranjana and H.S. Prakash, 2008. Molecular detection and characterisation of *Fusarium verticillioides* in maize (*Zea mays* L.) grown in southern India. *Annals of Microbiology*, 58(3): 359-367. 16pp, 497-516.
- Chandra, S.N., Shankar, A.C.U., Niranjana, S.R., Wulff, E.G., Mortensen, C.N. and Prakash, H.S. 2010. Detection and quantification of fumonisins from *Fusarium verticillioides* in maize grown in southern India. *World J Microbiol Biotechnol*, 26: 71-78.
- Chihaya, Y., Furusawa, Y., Okada, H., Matsukawa, K. and Matsui, Y. 1991. Pathological studies on systemic mycoses in calves. *Journal of Veterinary Medical Science*, 53(6). 1051-1058.
- Criseo, G., Bagnara, A. and Bisignano, G. 2001. Differentiation of aflatoxin-producing and nonproducing strains of *Aspergillus flavus* group. *Letters in Applied Microbiology*, 33: 291-295.
- Dall'Asta, C., Galaverna, G., Sforza, S., Biancardi, A., Dossena, A., Marchelli, R. New HPLC-FLD and HPLC-ESI-MS. 2003. methods for the simultaneous detection of type A and type B trichothecenes. In: Eklund, T.; De Brabander, H.; Daeseleire, E.; Dirinck, I.; Ooghe, W. (Eds.). *Strategies for safe food. Analytical, industrial and legal aspects: Challenges in organisation and communication. Euro Food Chem XII. Proceedings Vol. 2,*

- Koninklijke Vlaamse Chemische Vereniging, Heverlee, 2003: 608-612.
- Davis, N.D., Searcy, J.W. and Diener, U.I. 1969. Production of OA by *A. ochraceus* in a semisynthetic medium. *J. Appl. Microbiol.*, 17 (5): 742-744.
- Degola, F., Berni, E., Dall'Asta, C., Spotti, E., Marchelli, R., Ferrero, I. and Restivo, F.M. 2007. A multiplex RT-PCR approach to detect aflatoxigenic strains of *Aspergillus flavus*. *Journal of Applied Microbiology*, 103: 409-417.
- Färber, P., Geisen, R. and Holzapfel, W.H. 1997. Detection of aflatoxigenic fungi in figs by a PCR reaction. *International Journal of Food Microbiology*, 36: 215-220.
- Food and Agriculture Organisation (FAO, 2011). Global food losses and food waste: extent, causes and prevention. Rome, www.fao.org/fileadmin/user_upload/ags/publications/GFL_web.pdf
- Food and Drug Administration (FDA) 1994. Action levels for poisonous or deleterious substances in human food and animal feed. Washington, Department of Health and Human Services, 1125-126.
- Food and Drug Administration FDA 2000. Conference on mycotoxins in animal feeds, grains and food related to human and animal health. Rockville, Maryland.
- Gabal, M.A., Hegazy, S.M. and Nagwa, Y. Hassanien. 1994. "Aflatoxin production by field isolated of *Aspergillus flavus*." *Vet. Human Toxicol.*, 36: 519-521.
- Geisen, R. 1996. Multiplex polymerase chain reaction for the detection of potential aflatoxin and sterigmatocystin producing fungi. *Systematic and Applied Microbiology*, 19: 388-392.
- Geisen, R. 1998. PCR methods for the detection of mycotoxin-producing fungi. In: Bridge, P.D.; Arora, D.K.; Reddy, C.A.; Elander, R.P. (eds). *Application of PCR in mycology*. CAB International, New York, 1998: 243-266.
- Geisen, R., Mayer, Z., Karolewicz, A. and Färber, P. 2004. Development of a real time PCR system for detection of *Penicillium nordicum* and for monitoring ochratoxin A production in foods by targeting the ochratoxin polyketide synthase gene. *Syst Appl Microbiol.*, 27:501-507.
- Hamilton D. 2000. Toxic fungus threatens health of consumers. www.agnic.org/pmp/2000/ama0826.htm.
- Hassan A.A., Mogeda, K. Mansour, Howayda, M. El Shafei; Nouha, H.A., Oraby and Rasha, M.H. Sayed El Ahl . 2012. Studies on Mycosis and Mycotoxicosis in Cattle. *Bulletin of Environment, Pharmacology and Life Sciences*, Volume 1, Issue 3, February 2012: 12-22.
- Hassan, A. A., Rashid M.A. and Koratum Kh. M. 2008. Measurement of mycotoxins in feeds and sera of cattle and sheep and evaluation of its effect on some fertility related hormones in male rats. *Egypt. J. Comp. Path. & Clinic. Path.*, 21:340-358.
- Hassan, A. A., Wael M. Tawakkol, Abdel aziz A. El maaz and Howayda M. El Shafei, 2009. The hepatoprotective effect of dimethyl 4,4-dimethoxy 5,6,5,6-dimethylene di-oxy-biphenyl – dicarbxylylate (D.D.B.) against liver injury induced by aflatoxin B1 in rats. *Egypt. J. Appl. Sciences*, Vol. 24 No. (9), 86-100.
- Hassan, A.A. 2003. Detection of some mycotoxins and mycotoxins producing fungi in both macro- and microenvironment of diseased animals. 7th Sci. Cong. Egyptian Society for Cattle Diseases, pp. 112 – 119, 7-9 Dec., 2003, Assiut, Egypt.
- Hassan, A.A., M.A. Rashid Noha H. Oraby; S. El-ArabyKammal and M.M.Minshawy, 2013. Using of molecular biology techniques for detection of *Cryptococcus neoformans* in respiratory disorders in cows with references to its control by nanoparticles of iron oxide. *Egypt. J. of Appl. Sci.*, 28 (12) 433-448.
- Hassan, A.A., Nahed, M.El Mokhtar and Samira, A.M. Snousi. 2011. Studies on acute mycotoxicosis in turkey. *J. of New York Science J.*, 4 (5), 82-91.
- Hassan, A.A., Noha, H. Oraby, Aliaa A. E. Mohamed and Mahmoud H.H. 2014. The possibility of using Zinc Oxide nanoparticles in controlling some fungal and bacterial strains isolated from buffaloes. *Egypt. J. of Appl. Sci.*, 29 (3) 2014, 58-83
- Hassan, A.A., Wael M. Tawakkol; Elbrawy A.M. 2010. The hepatoprotective effect of dimethyl 4,4-dimethoxy 5,6,5,6-dimethylene dioxy-biphenyl - dicarbxylylate (D.D.B.) against liver injury induced by aflatoxin B1 in rats. *Int. J. Life Sciences*, Vol. 7 No. (3) (148-153).
- International Commission on Microbiological Specifications for foods (ICMSF) 1978. *Microbiology in foods, their significance and methods of enumeration*, 2nd Ed. University of Toronto press, Canada.
- Kamiya, A., Kikuchi, A., Tomita, Y. and Kanbe, T. 2005. Epidemiological study of *Candida* species in cutaneous candidiasis based on PCR using a primer mix specific for the DNA topoisomerase II gene. *J. Dermatol. Sci.*, 37 (1): 21-28.
- Keyle, A.C., A.N. Booth, M.S.Masri, M.R. Gumbmann and W.E., Gagne. 1970. Chronic effect of aflatoxin in farm animals feeding studies. Proceedings of the 1st. U.S-Japan Conference on toxic microbiology US. Dep. Of Interior . Washington, pp. 72-75
- Leaw, S.N., Chang, H.C., Sun, H.F., Barton, R., Bouchara, J.P., Chang, T.C. 2006. Identification of medically important yeast species by sequence analysis of the internal transcribed spacer regions. *J Clin Microbiol.*, 2006, 44:693–699.
- Lee, C.Z., Liou, G.Y. and Yuan, G.F. 2006. Comparison of the aflR gene sequences of strains in *Aspergillus section Flavi*. *Microbiology*, 152: 161-170.
- Leisová, L., Kucera, L., Chrpová, J., Sýkorová, S., Sip, V. and Ovesná, J. 2006. Quantification of *Fusarium culmorum* in wheat and barley tissue using real time PCR in comparison with DON content. *J. Phytopathol.*, 154: 603–611.
- Liewen, M.B. and Bullerman, L.B. 1999. Toxicogenic fungi and fungal toxins. In: Vanderzant, C.; Splittstoesser, D.F. (Eds). *Compendium of methods for microbiological examination of foods*. 3rd ed., American Public Health Association, Washington DC, 1999: 811-819.
- Meissonnier G.M., Pinton P., Laffitte J., Cossalter A.-M., Gong Y.Y., Wild C.P., Bertin G., Galtier P., Oswald I.P. 2008. Immunotoxicity of aflatoxin B1: Impairment of the cell-mediated response to vaccine antigen and modulation of cytokine expression. *Toxicol. Appl. Pharmacol.*, 2008; 231: 142–149.
- Mikulec, S.D., Stojanović, L. and Jovanović, L. 2005. Moulds associated with milk depending on Macroclimate and

- geographical location. *Applied Ecology and Environmental Research*, 3(2). 61-65. <http://www.ecology.kee.hu>.
- Mirhendi, H., Diba, K., Kordbacheh, P., Jalalizand, N. and Makimura, K. 2007. Identification of pathogenic *Aspergillus* species by a PCR-restriction enzyme method. *J Med Microbiol.*, 56, 1568-70.
- Morello, L.G., Sartori, D., Martinez, A.L.O., Vieira, M.L.C., Taniwaki, M.H. and Fungaro, M.H.P. 2007. Detection and quantification of *Aspergillus westerdijkiae* in coffee beans based on selective amplification of b-tubulin gene by using real-time PCR. *Int J Food Microbiol.*, 119:270-276.
- Morris, C. M., D. R. Ledoux, J. Broomhead, A. Bermudez, G. E. Rottinghaus, and A. Logan. 1997. Effects of pelleting on the toxicity of moniliformin in ducklings. *Poultry Sci.*, 76(Suppl. 1):15.
- Moubasher, A.H. 1995. Soil fungi are an active partner of our ecosystem. Their biodiversity and activities should be appreciated. *Qatar Univ. Sci. J.*, 15:239-247.
- Mullis, K.B. and Faloona, F.A. 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol.*, 155:335-345.
- Nesheim, S. 1971. Ochratoxin : occurrence, production, analysis and toxicity. 85 th. ANNM. Meeting AOAC. Washington, D.C.A. pp, 23.
- Ozaslan, M. İ., Caliskan, I. H., Kilic and I. D. Karagoz. 2011. Application of the ELISA and HPLC test for detection of aflatoxin in Pistachio. *Scientific Research and Essays*, Vol. 6(14), pp. 2913-2917, 18 July, 2011
- Parenicova, L., Skouboe, P., Frisvad, J., Samson, R., Rossen, L., Hoor-Suykerbyuk, M.T. and Visser, J. 2001. Combined molecular and biochemical approach identifies *Aspergillus japonicus* and *Aspergillus aculeatus* as two species. *Appl Environ Microbiol.*, 67:521-527.
- Paterson, R.R.M. 2006. Identification and quantification of mycotoxigenic fungi by PCR. *Process Biochemistry*, 41: 1467-1474.
- Patino, B., Gonzalez-Salgado, A., Gonzalez-Jaen, M.T. and Vazquez, C. 2005. PCR detection assays for the ochratoxin producing *Aspergillus carbonarius* and *Aspergillus ochraceus* species, *Int J Food Microbiol.*, 104: 207-214.
- Perrone, G., Susca, A., Stea, G. and Mule', G. 2004. PCR assay for identification of *Aspergillus carbonarius* and *Aspergillus japonicus*. *Eur J Plant Pathol.*, 110:641-649.
- Pitt JI and Hocking AD. 2009. Fungi and food Spoilage Book 4rd Ed., Chapter 4-10, pp. 29. Academic Press, New York, London, Toronto, Montreal, Tokyo.
- Polanco, A.M., Rodriguez, T. J. L. and Martinez, S. J. V. 1995. Detection of pathogenic fungi in human blood by the polymerase chain reaction. *Eur. J. Clin. Microbiol Infect. Dis.*, 14 (7): 618-621.
- Rahimi, P., Sharifnabi, B. and Bahar, M. 2008. Detection of Aflatoxin in *Aspergillus* Species Isolated from Pistachio in Iran. *J. Phytopathology*, 156, 15-20.
- Reddy, K.V., Naveen, K. and Reddy, I.B. 2013. Incidence and molecular detection of ochratoxigenic fungi from indian cereal grains. *Int. J. Pharm Bio. Sci.*, 2013 July; 4(3): (b) 31- 40.
- Refai, M. K. and Hassan, Atef, A. 2013. Monograph On Mycotoxigenic Fungi and Mycotoxins in Foods and Feeds with Synopsis on studies done by authors on mycotoxigenic fungi and mycotoxins in foods and feeds. <https://www.academia.edu/7688721-AtefHassan>. <https://www.researchgate.net/publication/AtefHassan>
- Richard J.L. 2007. Some major mycotoxins and their mycotoxicoses—An overview. *Int. J. Food Microbiol.*, 2007; 119: 3-10.
- Rotter, B.A., Prelusky, D.B. and Pestka, J.J. 1996. Toxicology of deoxynivalenol (vomitoxin). *J Toxicol Environ Health*, 48: 1-34.
- Saiki, R., Scharf, S., Faloona, F., Mullis, K., Horn, G.T., Ehrlich, H.A. and Arnheim M. 1985. Enzymatic amplification of b-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science*, 239:487-491.
- Sampietro, D.A., P. Marín, J. Iglesias, D.A. Presello, M.A. Vattuone, C.A. Catalan and M.T. Gonzalez Jaen. 2010. A molecular based strategy for rapid diagnosis of toxigenic *Fusarium* species associated to cereal grains from Argentina. *Fungal Biol.*, 114(1): 74-81.
- Samson, R.A., Houbraken, J.A.M.P., Kujipers, A.F.A., Frank, J.M. and Frisvad, J.C. 2004. New ochratoxin A or sclerotium producing species in *Aspergillus* section Nigri. *Stud Mycol.*, 50:45-61.
- Samson, R.A., Noomin, P., Meijer, M., Houbraken, J., Frisvad, J.C. and Varga, J. 2007. Diagnostic tools to identify black *Aspergilli*. *Stud. Mycol.*, 59:129-145.
- Sarlin, T., Yli-Mattila, T., Jestoi, M., Rizzo, A., Paavanen-Huhtala, S. and Haikara, A. 2006. Real-time PCR for quantification of toxigenic *Fusarium* species in barley and malt. *Eur. J. Plant Pathol.*, 114:371-380.
- Sartori, D., Furlaneto, M.C., Martins, M.K., Paula, M.R.F., Pizzirani-Kleiner, A., Taniwaki, M.H. and Fungaro, M.H.P. 2006. PCR method for the detection of potential ochratoxin-producing *Aspergillus* species in coffee beans. *Res Microbiol.*, 157:350-354.
- Scherm, B., Palomba, M., Serra, D., Marcello, A. and Migheli Q. 2005. Detection of transcripts of the aflatoxin genes aflD, aflO, and aflP by reverse-transcription-polymerase chain reaction allows differentiation of aflatoxin-producing isolates of *Aspergillus flavus* and *Aspergillus parasiticus*. *International Journal of Food Microbiology*, 98: 201-210.
- Schmidt, H., Ehrmann, M., Vogel, R.F., Taniwaki, M.H., Niessen, L. 2003. Molecular Typing of *Aspergillus ochraceus* and construction of species specific SCAR-primers based on AFLP. *Syst. Appl. Microbiol.*, 26:138-146.
- Schmidt-Heydt, M. and Geisen, R. 2007. A microarray for monitoring the production of mycotoxins in foods. *Int J Food Microbiol.*, 117:131-140.
- Smith, J.E., Lewis, C.W., Anderson, J.G. and Solomons, G.L. 1994. Mycotoxins in human nutrition and health. European Commission, Brussels, 1994, 300p.
- SPSS 14. 2006. "Statistical Package for Social Science, SPSS for windows Release 14.0.0, and 12 June, 2006. Standard Version, Copyright SPSS Inc., 1989-2006, All Rights Reserved, Copyright © SPSS Inc
- Van Quekelberghe, S., De Graeve, K. and Cordonnier, J. 2003. Simultaneous screening of 12 mycotoxins by Liquid Chromatography Tandem Mass Spectrometry. In: Eklund, T.; De Brabander, H.; Daeseleire, E.; Dirinck, I.; Ooghe, W. (Eds.). Strategies for safe food. analytical, industrial and legal aspects: Challenges in organisation and

- communication. Euro FoodChem XII. Proceedings Vol. 2, Koninklijke Vlaamse Chemische Vereniging, Heverlee, 2003: 706-709.
- Vos, P., Hogers, R., Bleeker, M., Reijans, M., Van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucl Acids Res.*, 23:4407-4414.
- Wafia, H. Abdallah and Hassan, A.A. 2000. Sanitary status of some ready to eat meat meals in Ciaro and Giza Governorates. *J. Egypt. Vet. Med. Ass.*, 60 (7): 95-104.
- Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.*, 18:6531-6536.
- Yang, X. and Quiros, C. 1993. Identification and classification of celery cultivars with RAPD markers. *Theor. Appl. Genet.*, 86: 205-212.
- Yong, R.K. and Cousin, M.A. 2001. Detection of moulds producing aflatoxins in maize and peanuts by an immunoassay. *Int. J. Food Microbiol.*, 65: 27-38.
