ORIGINAL ARTICLES

Molecular Detection of Fumonisin-producing *Fusarium* Species in Animal Feeds Using Polymerase Chain Reaction (PCR)

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ABSTRACT

Twenty one Fusarium isolates namely, *F. verticillioides* (15 isolates)*, F. proliferatum* (3isolates)*, F. anthophilum* (3 isolates), isolated from different types of animal feeds, collected from different animal farms in Cairo, Egypt, were subjected to PCR analysis. Depending on the data obtained from the random amplified polymorphic DNA (RAPD) analysis it was possible to discriminate between the three Fusarium species. The data indicated that 8 RAPD markers distinguished the *F. verticillioides* from the other species. Similarly 7 and 2 markers were found to be specific to *F. anthophilum* and *F. proliferatum* respectively. These markers can be considered as useful markers for proper identification of the three Fusarium species. In the present study a PCR-based detection kit for identifying fumonisin-producing *Fusarium* species was developed. One set of primers designed from the internal transcription spacer region (ITS) of the genus *Fusarium* was used. The data indicated that, all of the 21 isolates showed clear band corresponding to the molecular size of the ITS region (431bp), where it was absent in the control sample (*Aspergillus flavus*). The other set of primers specific to fumonisin producing *fum1* gene region of *Fusarium* was used to differentiate the fumonisin producing *Fusarium* species from non-fumonisin producers. The data indicated that all of the *Fusarium* isolates amplified fragments with the molecular size of 183bp corresponding to the correct size of *fum1* gene, while this band could not be detected in the control. The results of this study indicated that PCR-based technique could be used not only to differentiate the *Fusarium* species from other genera of fungi but also to identify fumonisin-producing *F. verticillioides, F. proliferatum* and *F. anthophilum.*

Key words: *Fusarium* species, Animal feeds, Fumonisin-producers, Molecular detection technique, Polymerase Chain Reaction.

Introduction

Fusarium species are important kernel rotting pathogens of cereals in subtropical and temperate regions of the world (Chulze *et al*. 1996). The *Fusarium* disease can colonize all portions of infected plants but infections, often symptomless, which have increased in the recent years due to ideal micro-climatic and physiological conditions provided by modern high yielding cultivars with a greater density of the plants per unit area accompanied by heavier nitrogen fertilizer (Silva *et al*. 2004). *Fusarium* infection was reported to occur systemically in all plant parts of maize (Desjardins *et al*. 2000)**.** In addition to damaging plant tissues some Fusarium species produce fumonisins (FBs), a group of mycotoxins as secondary metabolites.

Fumonisins are mycotoxins produced by at least 11 species of the fungus Fusarium, including the maize pathogens *Fusarium verticillioides* and *Fusarium proliferatum* (Fotso *et al*., 2002; Rheeder *et al*., 2002; Leslie *et al*., 2004; Yazar and Omurtag 2008; Proctor *et al*. 2008). Fumonisins are common contaminants of maize and are of concern because of epidemiological associations between ingestion of fumonisin contaminated maize and esophageal cancer and neural tube defects in some human populations (Hendricks, 1999). In addition, fumonisins can cause leukoencephalomalacia in horses, pulmonary edema in swine, and cancer and neural tube defects in experimental rodents (Marasas *et al*. 2004).

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Detection of fumonisin-producing fungal species by morphological characters sometimes is not enough for accurate identification of fungal isolates at the species level. Furthermore, both morphological and mating type characterization are time consuming and require considerable expertise in Fusarium taxonomy and physiology (Leslie and Summerell 2006; Jurado *et al*. 2010). As identification of Fusarium species is critical to predict the potential mycotoxigenic risk of the isolates, there is a need for accurate and complementary tools which permit a rapid, sensitive and reliable specific diagnosis of Fusarium species.

Therefore, improved and quicke methods such as DNA sequencing and species-specific PCR assay for identifying fumonisin forming fungi from animal feed staff has become important especially since fumonisins are now being implicated in diseases and cancer of animals. (Jurado *et al*. 2006a,b; Petrovic *et al*. 2009). Various PCR assays have been developed for the identification of toxigenic species of Fusarium. Some of them are based on single copy genes directly involved in mycotoxin biosynthesis while others are species-specific (Gonzalez Jae´n *et al*. 2004; Mule´ *et al*. 2005). The last ones often amplify multicopy target sequences, such as IGS or ITS regions (intergenic spacer and internal transcribed spacer of rDNA units, respectively), which increases the sensitivity of the assay in comparison with PCR assays based on single copy sequences. The use of these PCR approaches has been already useful in epidemiological analyses (Jurado *et al*. 2006a,b; Sreenivasa *et al*. 2008) and are widely used in fungal taxonomy (Baird *et al*. 2008; Chandra *et al*. 2010) and more recently they have been used for the detection of fungal pathogens in plant tissues (Chandra *et al*. 2008). Another important aspect of PCR techniques is that only small quantities of DNA are required to confirm the presence of a pathogen on a host tissue that otherwise might not be detected (Chandra *et al*.2008).

Molecular methods such as polymerase chain reaction (PCR) have been described to resolve genetic variation among isolates within or between formae speciales of *F. oxysporum* (Baird *et al*. 2008; 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 diagnostics of many fungal species (Annamalai *et al*., 1995; 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 have 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 intraspecific level. The rDNA repeat includes both highly conserved genes and more variable spacer regions.

The aims of this work were i) to identify and characterize toxin production of Fusarium isolates associated to animal feed from diverse regions of Egypt and ii) to evaluate the efficiency of DNA based tools to identify fumonisin producing Fusarium species in order to improve mycotoxin risk prediction in Egypt. The species-specific PCR assays used are targeted toward ITS sequences and on the toxin biosynthetic gene FUM1 (fumonisins).

Materials and methods

Collection of feed samples:

A total of 100 feed samples (200 g each), was collected for mycological examination. These samples included all types of feeds served to animals, namely barely, Soya bean and yellow corn. Samples were collected during the period from August 2008 to August 2009 from different equestrian centers in Cairo. Samples were brought to the laboratory using sterilized polyethylene bags and were stored at the refrigerator until examination.

Isolation of Fusarium species:

Of the aseptically mixed ground samples, 25 g were put into a stomacher jar containing 225 ml of peptone water. The homogenate sample was mixed by shaking and 1ml was transferred into a tube containing 9 ml of peptone water (1:10) and was mixed carefully by vortex. Several dilutions were made to obtain suitable number of colonies, which could be easily counted. After each dilution the solution was mixed thoroughly. One mililleter from each dilution was transferred into appropriately marked duplicate Petri-dishes containing 15-20 ml of Sabouraud's Dextrose Agar tempered to 45º C. The mixture was then thoroughly mixed and allowed to solidify. Inoculated plates were left to solidify at room temperature, then incubated at 25ºC for 5-7 days . During the incubation period, the plates were examined daily for the "Star-shaped" mould growth which is picked up under aseptic conditions with its surrounding cultivated medium and transferred into SDA slopes (Oxoid, 1990), then kept at 25ºC for 5-7 days, for purification and further identification. The incubated plates were examined visually and microscopically. The individual colonies of fungal isolates were selected depending upon their morphological characters and microscopic examination.

All Fusarium colonies were transferred onto Potato Dextrose Agar (PDA) and Carnation Leaf-Piece Agar (CLA) media to identify them up to the species level using fungal keys and manuals (Booth 1977; Leslie and Summerell ,2006).

Determination of Fumonisins in Feed Samples:

Fourteen feed samples (5 Soya, 5 corns, and 4 barely) from which 21 *Fusarium* strains were isolated, had been subjected to direct examination by fluorometric assay for determination of fumonisin content.

Molecular Detection of Fumonisin-producing Fusarium Strains:

DNA isolation:

DNA was isolated from 4 day growing fusarium mycelium that previously cultured in 500 µl Potato dextrose broth in 2 ml Eppendorf tubes and incubated at room temperature according to the method described by Zhang *et al*. (1998) with minor modification that is regularly being followed in our laboratory. The mycelial mat was pelleted by centrifugation at 5000 rpm (REMI C24 cooling centrifuge) for 5 min. The pellet was ground in microfuge tubes with blunt ends of disposable pipette tips in 500 µl of extraction buffer (2% CTAB, 1.4M NaCl, 20mM EDTA, and 100mM Tris-HCl, pH 8.0, pre-heated at 65°C) and incubated at 65°C for 20 minutes with gentle agitation. After the incubation period an equal volume of chloroform (500 µl) was added and mixed by vortex for one min. The supernatant was taken after centrifugation at 3000 rpm for 5 min at 4°C to a new tube and DNA was precipitated with an equal volume of ice-cold isopropanol, and incubated at -20°C for 60 min and again centrifuged at 8000 rpm for 8 min at 4°C. The pellet obtained was washed with 70% ethanol, air-dried, and re-suspended in 50 µl of nucleic acid free water and used directly for PCR.

To identify the fumonisin producing isolates at the molecular level, DNA samples from different fusarium isolates as well as from the control sample (*Aspergillus flavus*), were subjected to PCR analyses using fusarium species specific ITS and FUM1 primers. The PCR reaction was carried out in 25µl volume containing contained 10 ng of DNA sample, 10X Taq polymerase buffer(AB-gene Housse, UK), 25 mM $MgCl_2$, 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 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 primer sequence of both of the conserved *Fusarium* ITS DNA and the FUM 1 gene, used of this study was as follows (ITS Forward: 5'-AACTCCCAAACC CCTGTGAACATA-3' ITS Reverse: 5'- TTTAACGG CGTGGCCGC-3'), and (FUM1 F, 5'-CCATC ACAGTG GGACACAGT-3', FUM1 R, 5'- CGTATCGTCAGCATGATGTA GC-3') (Bluhm *et al*. 2004).

RAPD analysis:

In order to determine the genetic polymorphism between the isolated *Fusarium* species, genomic DNA was isolated from three isolates from each of *F. verticillioides, F. proliferatum* and *F. anthophilum* and were subjected to RAPD analysis. Five oligonucleiotide 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 *Fusarium* species 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 systat ver. 7 (SSPSS inc.c 1997 spss inc.3/97 standard version) computer programs were used to calculate the pairwise difference matrices (Yang and Quiros, 1993).

Results and discussion

The mould contamination, particularly with mycotoxin-producing fungi is a world-wide problem. The

fungal spores which affect horses predominantly originate from feed and fodder. It has been estimated that 25% of the world's crop production is contaminated with mycotoxins. This results in significant economic losses due to the loss of crops and animals. The present work was concerned with Fusarium contamination in animal feeds. Fusarium isolates were recovered from 23 feed samples out of 100 (23%), on the other hand Buckley *et al*. (2007) could detect Fusarium only in 2.6% of equine concentrated feed. Moreover, Khosravi *et al*. (2008) isolated Fusarium in 6% of animal feed mainly corn seed, barley and corn silage samples collected from Iran. The findings of Cvetnic *et al*. (2004) were much higher than ours as they found that *Fusarium* spp. were the most common fungi found in maize with the frequency of 78.6% in 1999 and 85% in 2003 at Croatia.

In the present study twenty-one *Fusarium* spp isolates recovered from animal feed stuff were subjected to morphological and microscopic examination. The data obtained revealed that these isolates were belongs to three different Fusarium species, namely *F. verticillioides*; *F. anthophilum* and *F. proliferatum* according to the fungal keys and manuals (Booth 1977; Leslie and Summerell ,2006).

Internal transcribed spacer (ITS) regions have been used successfully to generate specific primers capable of differentiating closely related fungal species (Bryan *et al*. 1995). In the broader context, taxon-selective amplification of ITS regions is likely to become a common approach in molecular identification strategies. Taxon-selective ITS amplification has already been used for detection of the fungal pathogens such as Fusarium (O´Donnell 1992; Siddiquee *et al*. 2010). In order to identify the 21 Fusarium isolates obtained at the molecular level, PCR analysis using primer specific for the conserved ITS DNA region of *Fusarium* genus was conducted. The data indicated that, all of the 21 isolates showed a clear band corresponding to the expected molecular size of the ITS region (431bp) and this band was absent in the control sample (*Aspergillus flavus*). These results confirmed that all the tested samples belong to the genus *Fusarium* (Fig.1).

In the present study the PCR analysis was employed to identify the Fumonisin producing fungi by analyzing the 21 *Fusarium* isolates using primers specific to the FUM 1 gene. The data indicate that all the tested samples of *F.verticillioides, F.proliferatum*, and *F.anthophilum* exhibited the FUM1 gene of Fumonisin-production and amplified fragments with the molecular size of 183 bp corresponding to the expected molecular size of the *fum1* gene sequence, while this band could not be detected in the control samples (*Aspergillus flavus*) (Fig.2). On the other hand, Sreenivasa *et al*. (2006) analyzed 32 isolates of *Fusarium* species by PCR for fumonisin producing ability using *fum 1* gene based primers, the expected DNA fragment of 183 bp was amplified only in 2 out of 12 isolates of *Fusarium moniliforme*, 3 out of 5 isolates of *Fusarium proliferatum*, and 3 out of 5 isolates of *Fusarium anthophilum*. In contrast, Sreenivasa *et al*.(2008) found that, when the isolates of *Fusarium* species were subjected to PCR analysis , the expected 183-bp DNA was amplified in all *F. verticillioides* (45/45), all *F. proliferatum* (04/04) and all *F. anthophilum* (04/04), respectively. Amplification was not detected in other isolates of *Fusarium* species (*F. pallidoroseum F. sporotrichioides F. oxysporum* and *Aspergillus flavus.*

Fig. 1: Agarose gel electrophoresis showing amplification of 431 bp. fragment of the internal transcription spacer region (ITS) of the genus *Fusarium*. Lane(1): DNA ladder(100 bp.) Lane(2): *Aspergillus flavus* (control negative) Lane (3-12): *Fusarium* isolates (samples)

Table 1: Detection of fuminisins in animal feed samples by fluorometric assay.

Feed stuff	Amount of fuminisins (ppm.) in animal feed samples *			
	Min.	Max.	Mean	± SE
Corn	4.7	40	18.62	13.14
Soya	1.4	36	15.56	14.58
Barley	24	34	28	4.32

Min = minimum , Max = maximum , SE = standard error ,*21 isolates were examined
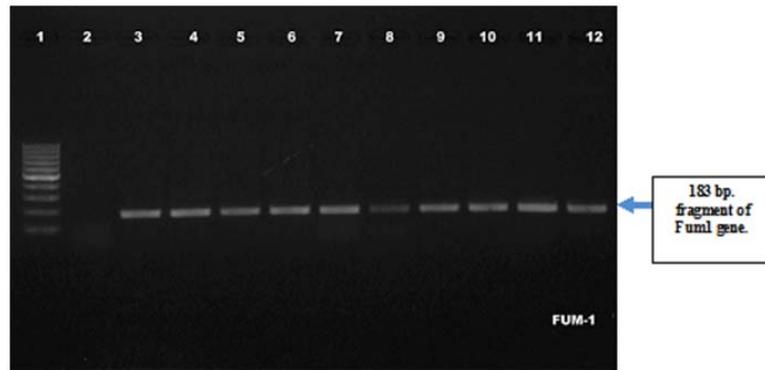
Fig. 2:   Agaose gel electrophoresis showing amplification of 183 bp. fragment of *fum1* gene. Lane(1): DNA ladder(100 bp.), Lane(2): *Aspergillus flavus* (negative control), Lane(3-7):*F.verticillioides*, Lane(8): *F.anthophilum* Lane(9-11): *F. proliferatum* and Lane(10-12): *F.verticillioides*

The fluorometric assay of fumonisin indicating that all tested feed samples contained fumonisin. The highest level of the fumonisin was recorded in barley, followed by yellow corn then soya samples Table (1). Sense the contamination of feed stuffs with different strains of moulds with special reference to *Fusarium* spp. and their toxins can cause many serious pathological problems for man and animals as already mentioned by several authors (Arin˜o *et al*. 2009; Orsi *et al*. 2009). The present data indicate that molecular detection of fumonisin producing fungi alone or in combination with fluorometric analysis is recommended to be done for the safety of animals.

In order to investigate the genetic polymorphism within and between three Fussarium species isolated from feed stuffs random amplified polymorphic DNA (RAPD) analysis was performed. Five out of twenty two primers tested resulted in the appearance of polymorphic and reproducible PCR products. This study showed that sixty one RAPD markers were detected between the three Fussarium species, of which 54 band were polymorphic (88.5%), while the remaining 7 band are common in all samples and can be considered as specific band for the genus Fusarium. The data presented in Fig (3) and Table (2) indicate that, 45% genetic polymorphism was detected among the *F. verticillioides* isolates while 64.4 and 55% genetic polymorphism was detected among the *F. anthophilum* and *F. proliferatum* isolates respectively (Table 2).

Table 2: RAPD primer names, equences, total and polymorphic bands generated within and between three *Fusarium* species

Primer name	Sequences	F. verticillioides		F. anthophilum		F. proliferatum	
		Total bands	Polymorphic bands	Total bands	Polymorphic bands	Total bands	Polymorphic bands
OPE-A-01	5'- CAGGCCCTTC -3'	9	7	7	4	10	7
OPE-A-03	5'- AATCGGGCTG -3'	8	0	11	4	8	1
OPE-A-10	5'- GTGATCGCAG -3'	11	4	11	9	9	9
OPE-C-02	5'- GTGAGGCGTC -3'	5	1	10	8	7	4
OPE-P-03	5'-CTGATACGCC -3'	7	6	6	4	6	1
	Total	40	18	45	29	40	22
	Polymorphism %	-	45	-	64.4	-	55

Table 3: The species-specific RAPD markers obtained from analyzing the three Fusarium species by RAPD analysis

Fusarium sp	Species specific RAPD markers	Total
F. verticillioides	A03-550, A03-200, A10-500,A10-380,A10-300, C02-450, P03-800, P03-200	8
F. anthophilum	A1-750,A03-900, A03-400, C02-1900, C02-250,P03-350, P03-300	7
F. proliferatum	C02-100, P03-500	2

The genotype-specific RAPD markers for the different Fusarium species used in the present study were determined (Table 3). The highest number of RAPD specific markers was scored for *F. verticillioides* (8 markers), followed by *F. anthophilum* recorded 7 specific markers, while *F. proliferatum* scored two markers. These markers can be considered as useful markers for proper identification of the three Fusarium species. These results agreed with finding reported by both of Abd-Elsalam *et al*. (2004) and El-Fadly *et al*. (2008), that molecular markers have proved to be useful, both in the identification of individual varieties and in the development of phylogenetic relationships among fungal species. Previously reported data indicated that, molecular techniques based on the polymerase chain reaction (PCR) have been used as a tool for genetic mapping, molecular taxonomy, evolutionary studies, and diagnosis of several fungal species (Welsh *et al*. 1991

and Sampietro *et al* 2010). RAPD analysis also offers several advantages that may be useful in studying races of *Fusarium oxysporum* (Nagarajan *et al* 2004; Bhim *et al*. 2006), and also to characterize strains of many *Fusarium* spp. (El-Fadly *et al*. 2008). The analysis of DNA products generated through random amplified polymorphic DNA (RAPD) has provided information on variation within and between Fusarium species which are in agreement with Lakhdar *et al*. (2004). The results of the present study revealed that PCR-based technique could be used not only to differentiate the *Fusarium* species from other genera of fungi but also to identify fumonisin-producing *Fusarium* species.
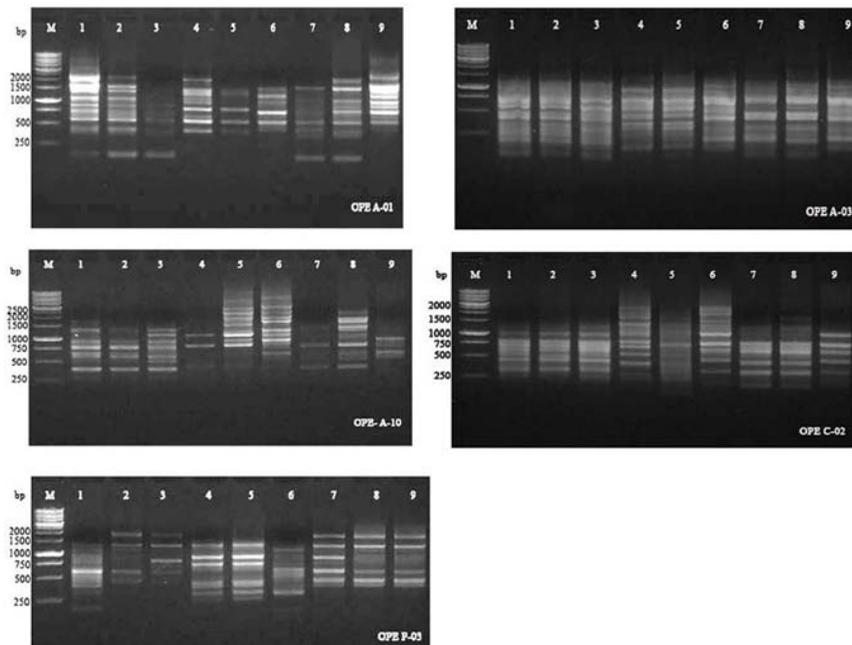

Fig. 3: RAPD banding patterns of nine Fusarium isolates recovered from animal feed stuff using five selected random primers, M: 1 kbp plus DNA ladder, Lane (1-3): *F. verticillioides*, Lane (4-6): *F. anthophilum* and lane (7-9): *F. proliferatum*

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