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Multiplex-PCR detection of fumonisin-producing *F.verticillioides* and *F.proliferatum* in corn of feedstuff

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Abstract

Cereals contamination of feedstuff such as corn with mycotoxigenic species of Fusarium is considered as a main source of fumonisin mycotoxin groups which cause worldwide serious diseases in human and animal health. According, in this study, a total of 100 Fusarium isolates were recovered from corn samples collected from 16 dairy farms in Razavy Khorasan Province, Iran, during 2011-2012. Fusarium isolates were identified on morphological and using species-specific PCR. Considering that, early detection of Fusarium species could be very important to prevent mycotoxin contaminating food and feed grains, a Multiplex-PCR assay was designed on the base of four sets of specific primers including: ITS-F/R, Vert1/2, FUM1-F/R and FP3-F/FP4-R which has been developed for the discovery of the two common Fusarium species (F.verticillioides and F.proliferatum) that they can potentially produce fumonisin. In this study, PCR analysis comfirmed 78% of morphological identification to species level as F.proliferatum (53.8%) and 29.5% as F.verticillioides. 42 (53.84%) of total (78) F.verticillioides and F.proliferatum isolated were fumonisin producing out of which 24 (57.14%) were F.proliferatum while 18 (42.86 %) were F.verticillioides. Moreover, we have established the method to discriminate between the two populations F.verticillioides and F.proliferatum, simultaneously enable us to discriminate fumonisin-producing strains from non-fumonisin-producing strains in the corn grains of animal feedstuff.

Keywords: Corn, Multiplex-PCR, *F.verticillioides*, *F.proliferatum*, fumonisin

Introduction

Corn (Maize) has been an important part of the feedstuffs. The fungus are distributed worldwide and infects cereal plants in all stages of their development, from the early hours of seeds germination to the time of harvest, including post-harvest decay of grains (Dass et al., 2007). Species of Fusarium are group of soil, saprobic and phytopathogenic fungi that have been found to produce fumonisins and other mycotoxins which responsible for acute diseases in human and animals such as, birth defects, alimentary canal irritations (Meng et all., 2010; Rheeder et al., 2002). Furthermore, the fumonisin group of toxins has, among others, mutagenic and thus carcinogenic properties (Galvano et al., 2002). *F.verticillioides* and *F.proliferatum* are the most prolific producer of fumonisins (Fandohan et al., 2003).

Traditional methods to identify Fusarium species related on the morphologic specifications of degenerate mycelia and reproductive organs such as spores (Meng et al., 2010). 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 (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 quick methods such as DNA sequencing and species-specific PCR assay for identifying fumonisin forming fungi from animal feedstuff has become important. 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 Jaen 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 (Jurado et al. 2006; Sreenivasa et al. 2008).

In this study, the Multiplex-PCR assays used are targeted toward internal transcribed (ITS) regions (between ribosomal genes) for the detection and identification of Fusarium spp. and the toxin biosynthetic genes FUM1 (fumonisins) and two sets of specific primers including: Vert1/2 and FP3-F/FP4-R. In this regard, the present investigation has combined the various individual PCR steps into a single diagnostic PCR assay (multiplex-PCR). Based on the approach presented in this work, a rapid and specific method has developed to detection *F.verticillioides* and *F.proliferatum,* simultaneously enable us to discriminate fumonisin-producing strains from non-fumonisin-producing strains in the maize grains of animal feedstuff.

Material and methods

Collection of maize samples

A total of 128 maize grain samples (1.5–5 kg) most commonly used in cattle feedstuff were obtained from 16 dairy farms (elected between both industrial and traditional types) in Razavy Khorasan Province, Iran, during four seasons 2011-2012 and 50 g of all 128 samples were stored from the date of collection at -18°C until analyzed.

Isolation and enumeration of fungal contamination in maize

At first, under aseptic conditions, each sample was homogenized, 10 gram of each finely ground maize sample were weighed and mixed with 90 ml of 0.1% sterile peptone water (0.1 g of peptone/100 mL of distilled water). The next, after 30 min of orbital shaking (100 rpm)

(Unimax 1010, Heidolph, Germany) followed by serial dilutions up to 10^{-5}. One ml volumes of each dilution in duplicate was deposited in a petridish (80 mm diameter), and the culture medium yeast extract chloramphenicol agar (YGC) (Merck, Danstadt, Germany) was poured over it. Plates were incubated in an upright position at 25°C. After 3, 5 and 7 days incubation, plates on which l0-150 colonies had formed were selected for separately enumerating molds. Total number of molds was determined by the dilution method. The colonies were counted and expressed as CFU per gram of samples (Pitt and Hocking, 1997). Individual fungal species were subcultured on the potato dextrose agar medium (PDA) (Sigma-Aldrich, USA) and were maintained at 4ºC and stored as spore suspensions in 15% glycerol.

Identification of Fusarium species

After determining the total number, in addition to morphological structure, mold colonies were subcultured on PDA, Carnation leaf-piece Agar (CLA), Malt extract agar (MEA) and Spezieller Nahrstoffarmer agar media (SNA) (Merck, Danstadt, Germany) and were identified them by slide culture, macro and microscopic characterization theirs. The type of sporulation and the morphology of the spores and spore-bearing structures are keys characteristics in fungus identification (Leslie and summerell, 2006). Determination of each species of fungi and mycological analyses were performed with using the keys and previously described (Klich and Pitt 1988; Pitt and Hocking, 2009) (Leslie and summerell, 2006). This was done by observing both macroscopic characteristics of the colonies on various media used as well as the microscopic morphology and measurements of the macroconidia, microconidia and conidiogenous cells, (after staining mycelia with 0.1% fuchsine dissolved in lactic acid) under an Olympus B061 Compound microscope (Wirsam Scientific, S. Africa) and microscope equipped with a Dino Camera Ser. No. 208060245 and Dino capture software (Zeiss, West Germany).

DNA extraction and amplification of Fusarium species

The Fusarium colonies isolated were grown on PDA plates. Three mycelia disks inoculated in to 100 ml sterile potato dextrose broth, then were incubated for 5-7 days at room temperature under shaking conditions (180 rpm) and later dried with freezer-dried. Total DNA was extracted from grinded mycelium of each isolate (~20-50 mg) using a AccuPrep Genomic DNA Extraction Kit (Bioneer, korea). Also a CTAB DNA extraction procedure was performed according to a modified method of Noorbakhsh et al. (2009). In order to do this, lysis buffer [2% CTAB, 1 M Tris-HCL pH 8.0, 0.05 M ethylenediaminetetraacetic acid (EDTA), 1% (w/v) sodium dodecyl sulphate (SDS), 0.9 M sodium choloride (NaCl), 0.1 M sodium sulphite (Na2SO3)] was added and heat-shock treated at 65ºC for 20 min. The suspension was centrifuged (Fisons, Loughborough, England) for 5 min at 2,000 g and the supernatant was transferred to a new microfuge tube. The supernatant was then extracted with chloroform: isoamyl alcohol (24:1). The solution was placed -20ºC for 4 hours and then centrifuged for 10 min at 2,000 g. After centrifugation, the supernatant was taken to a new microfuge tube. The DNA was precipitated with equal volumes of isopropanol alcohol and centrifuged for 5 min at 2,000 g. After centrifugation, the supernatant was removed and the pellets obtained were resuspended in 100 µL distilled water for PCR analyses.

Moreover, to confirm of identification the fumonisin-producing isolates, 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.

Determination of fumonisin-producing Fusarium by Multiplex-PCR

All genomic DNA obtained of each strain were tested for Multiplex PCR amplification using specific primers ITS Forward (5'-AACTCCCAAACCCCTGTGAACATA-3') and ITS Reverse (5'-TTTAACGGCGTGGCCGC-3'), which amplify the ITS region in Fusarium Species with PCR product of 431 bp (Ben Amar et al., 2012). The primer pair specific to *F.verticillioides*: VERT-1 (5'-GTCAGAATCCATGCCAGAACG-3') and VERT-2 (5'-CACCCGCAGCAATCCATCAG-3)' and the expected amplicon size was ~800 bp (Maheshwar et al., 2010).

Additionally, a pair of primers specific to *F. proliferatum* was used: Fp3-F (5'-CGGCCACCAGAGGATGTG-3') and Fp4-R (5'-CAACACGAATCGCTTCCTGAC-3'), which amplified a fragment of ~230 bp (Jurado et al., 2006). As well as, another set of primers, FUM1 forward (5′-CCATCACAGTGGGACACAGT-3′) and FUM1 reverse (5′-CGTATCGTCAGCATGATGTAGC-3′) was used to determine the fumonisin. Protocol was developed based on primers specific for producing ability of *F.proliferatum* and *F.verticillioides*. The expected amplicon size for FUM1 was 183 bp (Bluhm et al. 2004) (Maheshwar and Janardhana, 2010). The primers were purchased from Bioneer Corporation (Korea).

Table1: Primers used in this study. The expected product sizes are ideal for multiplex PCR assay.

Target species	Primer designation	Sequence[1]	Fragment Size(bp)	Ref
Fusarium genus	ITS Forward ITS Reverse	5'-AACTCCCAAACCCCTGTGAACATA-3' 5'-TTTAACGGCGTGGCCGC-3'	431	Ben Amar et al., (2012)
F. verticillioides	VERT-1 VERT-2	5'-GTCAGAATCCATGCCAGAACG-3' 5'-CACCCGCAGCAATCCATCAG-3'	~800	Maheshwar et al., (2010)
F. proliferatum	FP3-F FP4-R	5'-CGGCCACCAGAGGATGTG-3' 5'- CAACACGAATCGCTTCCTGAC-3'	~230	Jurado et al., (2006)
fumonisin producing	FUM1-F FUM1-R	5'-CCATCACAGTGGGACACAGT-3' 5'-CGTATCGTCAGCATGATGTAGC-3'	183	Bluhm et al. (2004)

[1]- Sequence specificity all of the primers was checked by blastn program. (http://www.ncbi.nlm.nih.gov/BLAST/).

Amplification reactions were carried out in volumes of 25 µl containing 2 µl of template DNA, 1 µl of each primer (20 pmol), 0.2 µl of Taq DNA polymerase (3 U/µl), 2.5 µl of 10X PCR buffer, 0.75 µl of 50 mM MgCl2, and 0.5 µl of 10 mM dNTPs (Bioneer Corporation, Korea).

The goal of our study was to optimize the multiplex PCR instead of only optimizing PCR detection of individual genes. In order that, primers were combined and various annealing temperatures as well as primer concentrations were also tested. Negative and positive controls were included while performing PCR.

This protocol was optimized by determining the common annealing temperature at which each of the primers (Table1) amplified the target genes without any nonspecific fragment amplification. Finally, annealing temperatures between 50 and 62ºC were tested and for the multiplex PCR, determining primer concentrations in the reaction was critical.

The PCR Reactions were performed in a PCR system thermal cycler (Sensquest, Germany) using the following PCR conditions: initial denaturation for 5 min at 95ºC, followed by 35 cycles of denaturing (95ºC for 60 s), annealing (50-60ºC for 60 s), and primer extension (72ºC

for 30-60 s). Amplification was terminated after a final extension period of 72ºC for 7-10 min, followed by cooling at 4°C until recovery of the samples. From the results, it was evident that annealing temperature of 55ºC worked the best.

Amplification products were visualized in 2% agarose gels stained with ethidium bromide in 1X Tris-acetate EDTA buffer for 120 min at 80 V (Mule et al., 2004). Gel images were captured using GEL DOC Vilber Lourmat Imaging System (Vilber Lourmat, Astralia) (version D10374-CAT) software.

Statistical analysis

IBM SPSS Statistic 20 software was used for statistical analysis. Mold's total counts were logarithm transformed. Analysis of variance (ANOVA) was done and Tukey test at 5% significance level was used to compare the mean of total count in different season.

Result

Morphological identification of fungal contamination in maize samples

Based on the results obtained, mold's total count range was 5-5.5×10^5 CFU per gram of maize grain samples. Fusarium genera were found in all of the samples with the highest frequency. According to morphological characters, the most predominant fungal genera in maize samples were included: *Fusarium spp*. (36.94%), *Penicillium spp.* (27.7%), *Aspergillus spp.* (21.16%), *Mucor spp.* (3.64%), *Monilia spp.* (1.43%), *Cladosporium spp.* (0.28%), *Alternaria spp.* (0.24%), *Geotrichum spp.* (0.14%). Similar to the findings reported by Karnajaja, et al. (2008), in their study,the most prevalent fungi genus was Fusarium (100 and 100%) followed by Aspergillus (73.94 and 87.10%), Penicillium (45.78 and 70.32%), Mucor (37.32 and 33.55%) while the least frequent species were from genus Alternaria, 4.93% and 16.13% in 2007 and 2008, respectively. These findings were also supported by Njobeh et al., (2009) and Kranjaja et al., (2009) study.

As well as, the data obtained revealed that Fusarium isolated were belongs to two mainly Fusarium species, namely *F.verticillioides* and *F.proliferatum* according to the fungal keys and manuals (Leslie and Summerell, 2006). This finding is in good agreement with the earlier studies, which reported the most of Fusarium species were *F.proliferatum* and *F.verticillioides* from maize (Ghyasian, et al., 2011), (Fandohana, et al., 2005).

The mold total count reported in this study. Based on ANOVA and Tukey analysis test, there was significant different among seasons. As a result autumn season has the highest fungal contamination (13.5 ×10^5 CFU/gram) and while the lowest levels of contamination was related to summer season (2.82 ×10^5 CFU/gram). Moreover, there was no significant difference between winter and spring. During autumn, from maize samples for dairy cow, the widest fungi species was isolated and the most present was related to species of Fusarium. These results were also obtained in Krnjaja et al., (2009) study.

Multiplex-PCR for identification of fumonisin-producing

Of the 100 *Fusarium* isolates identified based on morphological criteria, all isolates scored positive for the ITS region. On the other hand, all isolates were tested with the species-specific primers VER1/2 and Fp3-F/Fp4-R that, 78 strains were confirmed to species level with species-specific PCR amplification as *F.proliferatum* (53.8%) and 29.5% as *F.verticillioides*. Further, all of the isolates were tested with species-specific primer pairs FUM1-F and FUM1-R, according to data from electrophoresis of PCR products, 42 (53.84%) of total (78) *F.verticillioides* and *F.proliferatum* isolated were fumonisin producing out of which 24 (57.14%) were *F.proliferatum* while 18 (42.86 %) were *F.verticillioides.* Two agarose (2% w/v) gels in figure 1 and 2 show several Fusarium DNA samples at 100 ng that

were amplified by multiplex PCR. Single bands were obtained with species-specific primers from all strains belonging to *F.proliferatum* and *F.verticillioides.* This multiplex PCR method based on FUM1 gene and species-specific primers allowed detection and discrimination of the fumonisin producers *F.proliferatum* and *F.verticillioides* simultaneously.

Iin this regard, Bluhm et al. (2002, 2004) developed a multiplex PCR assay for the simultaneous detection of fumonisin (using polyketide synthase, FUM1 gene) and trichothecene producing Fusarium spp. in cornmeal. The FUM1 optimized primer concentration in the multiplex PCR was consistent with findings of Bluhm et al. (2002). Also in Sanchez-Rangel et al. (2005) study, a PCR-based method, to detect a region of the FUM1 gene involved in fumonisin biosynthesis, was developed and employed to detect mycotoxigenic fungi in pure culture and in contaminated maize. They showed the usefulness of these primers in such an application but moreover, in this study, was demonstrated the added advantage of using the elongation factor species-specific primers that a well established database is available for identification of Fusarium species obtained by this research.

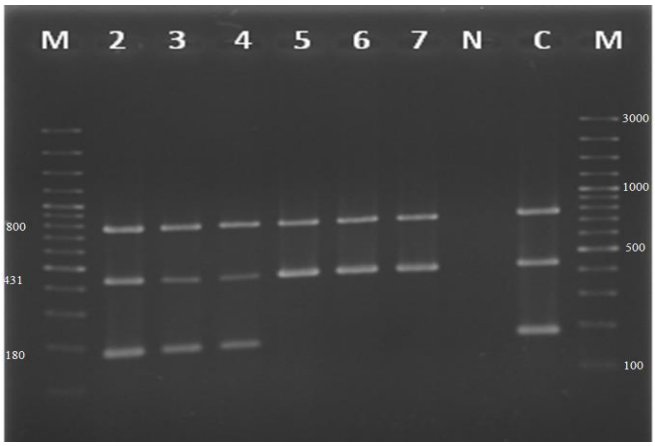

Figure-1: A 2% (w/v) agarose gel depicting the successful multiplex PCRs in which ITS-F/R: 431 bp primers, VERT-1/2:~800 pb and FUM1-F/R: 183 bp primers were used. Lane M is 100 bp DNA ladder; Lanes: 2-7 *F. verticillioides* isolated by morphological criteria, Lan N: *Aspergillus flavus* as negative control, Lan C: positive control: *F. verticillioides* (ATCC Number: 204499)

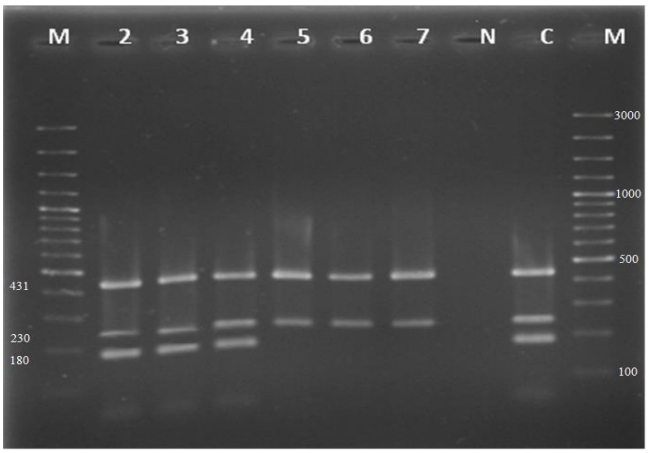

Figure-2: Agarose gel 2% (w/v) showing amplified products of multiplex PCRs with three primers including: ITS-F/R: 431 bp, Fp3-F/ Fp4-R: 230 pb and FUM1-F/R: 183 bp. Lane M is 100 bp DNA ladder; Lanes 2-7 *F.proliferatum* by morphological criteria, Lan N: *Aspergillus flavus* as negative control, Lan C: positive control: (ATCC Number: 204497)

Discussion

The mold contamination, particularly with mycotoxin-producing fungi is a world-wide problem. The fungal spores which affect domestic animals predominantly originate from feed and fodder. It has been estimated that 25% of the world's crop production is contaminated with mycotoxins which causes important economic losses due to the loss of crops and animals (Abo-El-Yazeed et al., 2011).

The genus Fusarium contains a diverse array of fungi, members of which are phytopathogenic to a wide range of plants and cereals under diverse environmental conditions. Torres, et al. (2001) suggested that the climatic conditions under which cereals (as corn grains) are stored could have an important influence in growth the spread of the fungal species such as Fusarium. Also, mycotoxin production in grain can begin in the field and continue throughout storage. Most species produce spore, grow best, and are most pathogenic to cereals at optimal temperatures (15–25ºC) and under humid conditions. In Iran, corn is one of the most important crops imported annually. However, only limited data are available on the fungal and mycotoxin contamination of Iranian corn and imported on the ability of local fungal strains to produce mycotoxins.

The present work, Fusarium isolates were recovered from 36.94% maize grain samples in feedstuff Collected from farms. According to the current measurements, the most of Fusarium species of corn samples were belonging to *F.proliferatum* and *F.verticillioides.* Khosravi et al. (2008) isolated Fusarium in 6% of animal feed mainly corn seed and corn silage samples collected from Iran. Moreover, Buckley et al. (2007) could detect Fusarium only in 2.6% of equine concentrated feed. The findings of Cvetnic et al. (2004) *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 this research, according to obtained results in different seasons, autumn had the highest fungal contamination (13.5×10^5 CFU/gram). In this regard, study of Doohan, et al., (2003) indicated that increased levels of fungal growth and production of toxins as fumonisins were related to elevated regional temperatures. Furthermore, the association of high temperatures is a factor that influences fungal growth and subsequent deleterious mycotoxin production (Homdork et al., 2000). In fact, during autumn with optimal humidity and temperatures, cereals localized in environments were more susceptible for growth of Fusarium spp.

Since investigation on the presence and protection of mycotoxigenic fungi on feedstuffs are very important and also corn is one of the main components of cattle feed, therefore continuous and consecutive seeking on contaminating fungal species and their accurate detection is of paramount importance to take the appropriate preventive measures.

In summary, the multiplex-PCR based method used in the present study was found to be quick and more sensitive in identification of *F.verticillioides* and *F.proliferatum* isolates, in comparison with agar plating method. In addition, this approach provides evidence that primers ITS-F/R, Fp3-F/Fp4-R, VERT-1/2 and FUM1-F/R described could be useful tools for rapid detection and identifying mycotoxigenic Fusarium species in food and feedstuff samples. When the primers were combined in this multiplex-PCR, three fragments of appropriate sizes were obtained. This demonstrates the potential of using these fragments in a multiplex-PCR that aims at detection of fumonisin positive Fusarium along with the identification of pollutant species. Therefore this study may be faster than culture-dependent methods and could especially be of value for the rapid detection of slow growing toxin-producing species in food samples.

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