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Multiplex PCR Assay for the Detection of Aflatoxigenic and Non-Aflatoxigenic *Aspergilli*

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Abstract: Aflatoxins are potent secondary metabolites produced commonly by *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. Primers were designed specifically for *o-methyl transferase* (*omt*) and aflatoxin regulatory gene (*aflR*) of aflatoxin biosynthetic pathway and also to detect the genus *Aspergillus* specific primers (18*s* rRNA genes) using *NS*. Experimental conditions were standardized for optimum multiplex PCR. DNA extracted from mycelia of toxigenic and non-toxigenic *A. flavus*, *A. parasiticus*, other *Aspergilli* and from other genera of fungi were subjected to multiplex PCR using these primers. The *omt* and *aflR* primer pairs gave specific PCR amplification for aflatoxigenic *A. flavus* and *A. parasiticus*. They did not give DNA amplification for non-aflatoxigenic *A. flavus*, *A. oryzae*, *A. glaucus*, *Fusarium*, *Penicillium* and *Rhizopus* spp.

Key words: Aflatoxin, *A. flavus*, *A. parasiticus*, PCR, Multiplex PCR, *aflR*, *omt*, *NS*

INTRODUCTION

Many mycotoxins have been shown to be toxic, carcinogenic, mutagenic in nature. Contamination of the food with mycotoxins is a major problem throughout the world and removal of mycotoxins from contaminated food is difficult and costly (Marasas *et al*., 2001). About 25 to 50% of the crops harvested worldwide, are contaminated with aflatoxigenic fungi. In India 25-50% food and feed have been reported to be contaminated by mycotoxigenic fungi during post harvest operations and storage. The contaminated agricultural commodities will always have a heterogeneous mixture of toxigenic and non-toxigenic species of fungi. The identification and differentiation between toxigenic and non-toxigenic fungi is based on morphological characteristics and cultural characteristics in specific media. This type of identification is tedious and requires extensive training and expertise. Therefore a rapid and reliable assay for routine differential identification of toxigenic and non-toxigenic moulds would benefit the agricultural industry.

Inspite of advances in analytical methods of detection of aflatoxins, these advanced physico-chemical methods have some disadvantages. They need highly elaborate and sophisticated clean-up and/or derivatization procedures (Smith *et al*., 1994). Much simpler and faster immunochemical methods have the disadvantage to follow the concept of one substance one assay (Smith *et al*., 1994; Young and Cousin, 2001). The identification of fungi associated with agricultural commodities has been advanced by the development of PCR assay to detect specific sequences of DNA unique to the toxigenic strains. The usefulness of PCR methods to monitor quality and safety of agricultural commodities can be well exploited to differentiate the toxigenic and non-toxigenic strains. Multiplex

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PCR is used for simultaneous amplification of multiple targets/loci and is applied as a diagnostic tool to detect multiple gene mutations (Uggozzoli *et al*., 1998) and for simultaneous detection of infectious agents (Jungkind *et al*., 1996). A single assay combining the detection of genus-specific *Aspergilli* along with aflatoxigenic species would be of advantage in estimating the extent and type of contamination of any agricultural commodity.

In this communication we report a multiplex PCR assay to detect non-aflatoxigenic and aflatoxigenic species of *Aspergilli* simultaneously.

MATERIALS AND METHODS

Taq polymerase, dNTPs were purchased from Bangalore Genei, Bangalore, India. Other chemicals used in these studies were of molecular biology grade and purchased from standard chemical companies. The primers were designed specifically for aflatoxin regulatory gene (*aflR*), o-methyl transferase gene (*omt*) of aflatoxin biosynthetic pathway (Yu *et al*., 1993) and 18*s* small nuclear ribosomal region (*NS*) (White *et al*., 1990), using primer 3 software. The primers were designed based on the published sequence strand for *A. flavus* and *A. parasiticus* from the NCBI databank by the authors and has been patented (Manonmani *et al*., 2002). With the assumption that *NS* is unique to the genera *Aspergilli* and *omt* and *aflR* unique for aflatoxigenic fungi, in present studies, all these primers were used in PCR reaction.

Standard toxin was purchased from Sigma- Aldrich Chemical Company, USA.

Fungal Strains

The fungal species used in this study are shown in Table 1. The isolates were maintained on potato-dextrose agar or on Czapek-Dox Agar media. The cultures were sub cultured periodically and 5 day old slant cultures were used in these studies.

Table 1: Fungal isolates used for multiplex PCR

Fungal isolate	Source	Mycotoxin
A. flavus ATCC 46283	Groundnut seeds	Aflatoxin B_1 and B_2
A. ochraceus CFR 221	Coffee curing premises	Ochratoxin A
A. flavus NCIM 645	Standard strain	Aflatoxin B_1, B_2, G_1 and G_2
A. flavus MTCC 152	Standard strain	AflatoxinB_1,B_2, G_1 and G_2
A. flavus	Japan	Non-toxic
A. flavus	Corn	Non-toxic
A. flavus	Millet	Aflatoxin B_1 and B_2
A. parasiticus CFR 223	Groundnuts	Aflatoxin B_1,B_2, G_1 and G_2
A. flavus	Wheat	Non-toxic
A. flavus	Maize	Non-toxic
A. oryzae	Curry spice mix	Non-toxic
A. oryzae	Food	Non-toxic
A. oryzae	Meatcurry spice mix	Non-toxic
A. oryzae CFR 225	Soil	Non-toxic
A. glaucus	Food	Non-toxic
Aspergillus spp.	Peda-milk sweet	Non-toxic
A. flavus	Food	Non-toxic
A. niger CFR 224	Soil	Non-toxic
P. verrocosum MTCC 2007	Standard strain	Non-toxic
Fusarium spp.	Tomato	T-2 toxin
Fusarium spp.	Traditional starter-*Manapu*	Non-toxic
F. tricinctum NRRL 32998	Standard strain	T-2 toxin, Diacetoxyscirpenol (DAS)
F. fujikuori NCIM 665	Standard strain	Deoxynivalenol (DON) and T-2 toxin
F. saubinetti NCIM 851	Standard strain	Deoxynivalenol (DON) and T-2 toxin
Fusarium spp.	Chow Vegetable	Zearalenone
Rhizopus arrhizus NCIM 997	Standard strain	Non-toxic
Rhizopus chinensis	Traditional starter *Manapu*	Non-toxic

Screening of Fungal Strains for Aflatoxins

All the fungal strains belonging to *A. flavus* and *A. parasiticus* were screened for the production of aflatoxins using YES medium. YES medium (yeast extract 20 g, sucrose 150 g, distilled water 1 L, pH 4.5) was inoculated with spore suspension of standard/test samples. The flasks were then incubated at room temperature for seven days under dark and stationary conditions. Aflatoxin in the broth medium was extracted and estimated by Pon's modified method (Rati *et al.*, 1987). Aflatoxin extract was purified on silica gel column, derivatized with trifluoroacetic acid- Aflatoxin B_1, G_1, B_2, G_2, separated by reverse-phase liquid chromatography and detected by fluorescence using a fluorichrom fluorescence detector (varian) with 360 nm excitation and 440 nm emission. The LC column used for separation was Supelcosil LC-18, 15×4.6 mm id, mobile phase was water: acetonitrile: methanol:: 70:17:17. This method can measure 0.1 ng of aflatoxins B_1, B2, G_1 and G_2. The test samples were quantified for aflatoxin by comparative calculation with the standard peak area values (Scott, 1995).

Isolation of Fungal DNA

Template DNA was extracted from fungal mycelia according to Lee *et al.* (1998) (individual fungal isolates or their mixtures or from enrichment of food samples) as follows: fungal mycelia grown in Potato Dextrose Broth (PDB) under stationery conditions for 21 days was harvested by filtration. The mycelium was washed twice with phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) followed by centrifugation. The mycelium was transferred to a mortar and ground well. Freshly prepared, sterile Lysis buffer (50 mM Tris, 150 mM EDTA, 1% (w/v) SDS, pH 8.0) was added to the pulverized mycelia and incubated at 65°C for 1 h. The suspension was centrifuged and supernatant was then extracted twice with phenol: chloroform: isoamylalcohol (25:24:1) and the aqueous layer was washed twice with chloroform and then precipitated with two volumes of isopropanol. The precipitate was resuspended in 200 μL of TE buffer (10 mM Tris-Cl, 1.0 mM EDTA, pH 8.0) (Lee *et al.*, 1998).

Multiplex PCR

All the primers were synthesized by Sigma genosys, UK. The PCR conditions were optimized by varying the concentration of these three primer sets, the number of units of Taq polymerase and annealing temperature of the reaction. The PCR reaction mixture (25 μL) contained 100 ng of genomic DNA, deoxyribonucleoside triphosphates at 0.025 nmol each, primers at 4 nmol each and reaction buffer. Each reaction mixture was heated to 95°C for 10 min before adding 0.3 units of Taq DNA polymerase. Amplification conditions used consisted of 4 min at 94°C followed by 35 cycles at 94°C for 30 sec, 50°C for 45 sec, 72°C for 75 sec. The reaction was completed with incubation for 10 min at 72°C. PCR products were analysed by electrophoresis in a 1% agarose gel in TAE buffer. Ethidium bromide (0.5 μg μL^{-1}) stained gels were visualized under UV light and documented in a gel-doc system with CCD camera attached to it (Hero-Lab), (Sambrook and Russell, 2001).

RESULTS AND DISCUSSION

PCR Primers

In this study we designed primers from *omt* gene, which is involved in the conversion of sterigmatocystin to o-methylsterigmatocystin of aflatoxin biosynthetic pathway, *alfR* gene, which is involved in the regulation of aflatoxin biosynthesis, for use in PCR assay. The *NS* primer set was from 18s rRNA region uniquely homologous to *Aspergillus* species (White *et al.*, 1990). The *omt* and *aflR* primer sets were obtained from conserved regions reported for *omt* and *aflR* genes (Yu *et al.*, 1993). On the basis of the design methodology, a single assay should result in the detection of *Aspergillus* genus as well as aflatoxin producing species. The main purpose was to combine the three primer sets

Table 2: Fungal species analysed by multiplex PCR

Fungal species	Detection of PCR product with primer set		
	omt	*aflR*	*NS*
A. flavus ATCC 46283	+	+	+
A. parasiticus CFR 223	+	+	+
A. flavus NCIM 645	+	+	+
A. flavus MTCC 152	+	+	+
A. flavus	+	+	+
A. oryzae	-	-	+
A. niger CFR 224	-	-	+
Fusarium spp.	-	-	-
Rhizopus spp.	-	-	-
Penicillium spp.	-	-	-
A. ochraceus CFR 221	-	-	+

+: Positive PCR amplicon, -: Negative for PCR amplification

into a single PCR reaction. A key advantage of combining these primers in a single reaction is that the presence or absence of genus specific band at 555 bp corresponding to *NS* region serves as an internal control for genus specific identification and *omt* and *aflR* amplified band serves in the specific detection of aflatoxigenic species. This serves in the detection of the extent of contamination and contaminant type of any food and feed sample. In the earlier work the PCR amplification product of *alfR* gene primer was confirmed by restriction digestion. PCR amplicons of *A. flavus* and *A. parasiticus* were subjected to restriction endonuclease (*Hinc* II and *Pvu*II) analysis to differentiate the two species with specific RFLP's (Somashekar *et al*., 2004).

Amplification of Aflatoxigenic *Aspergillus* spp.

To test the specificity of the designed primer sets in a PCR reaction, other members of *Aspergilli*, viz., *A. oryzae*, *A. niger*, *A. ochraceus* and other genera of fungi such as *Fusarium* spp., *Rhizopus* spp. and *Penicillium* spp. were evaluated (Table 2). The *NS* primer pairs were highly specific for the genus *Aspergilli* (Fig. 2, lanes 1, 2 and 4). All the aflatoxigenic *Aspergilli* gave positive amplification with both *omt* and *aflR* primer pairs (Fig. 1, lanes 4, 5, 6, 7 and 8). Non-aflatoxigenic *Aspergilli* screened did not show amplification with *omt* and *aflR* primer pairs indicating the absence of aflatoxin producing machinery. However, the *Aspergillus* genus specific *NS* primer pair gave positive amplification and other fungal cultures did not show amplification with these primer pairs. This indicated the specificity of *NS* primer pairs with the genus *Aspergillus* and *omt* and *aflR* primer pairs with aflatoxigenic *Aspergilli*.

Production of Aflatoxin

All the fungal isolates screened by multiplex PCR were screened for toxin production in YES medium (Table 3). Aflatoxin B_1 was found to be produced by *A. flavus* while *A. parasiticus* produced all the four toxins, B_1, B_2, G_1 and G_2. Some of the *A. flavus* isolates did not produce any of the toxins on YES medium. The HPLC analysis showed a clear differentiation between aflatoxin producing and non-aflatoxin producing strains of *A. flavus* (data not shown).

The multiplex PCR developed using three sets of primers for *omt*, *alfR* and *NS* showed positive correlation for aflatoxin production where a complete pattern with three bands was obtained on agarose gel (Fig. 1). For non-aflatoxigenic producing *Aspergilli*, only one band corresponding to *Aspergillus* specific 18s rRNA region was obtained (Fig. 2). The multiplex PCR could be used as a marker to clearly differentiate between the aflatoxin-producing and non-aflatoxigenic *Aspergilli*. In a similar kind of work (Criseo *et al*., 2001) studied the differentiation between aflatoxin producing and non-producing *A. flavus* group. They studied quadruplex-PCR using *aflR*, *nor-1*, *ver-1* and *omt4* gene

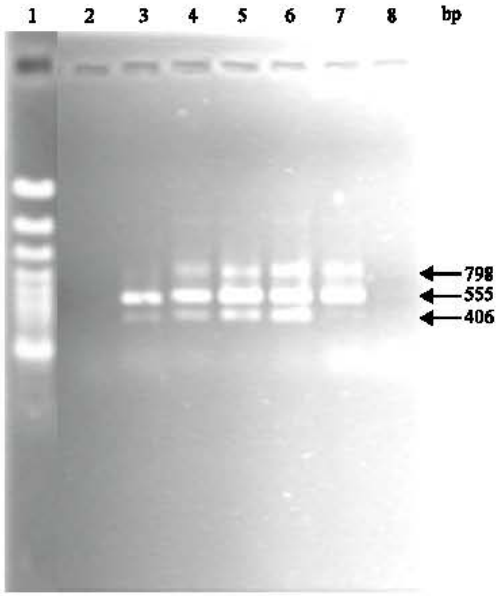

Fig. 1: PCR amplification of *omt* and *afl* R genes from different fungal species. Lane No. (1) Marker 1000 bp, (2) *Rhizopus*, (3) Environmental control, (4) *A. flavus* ATCC 46283, (5) *A. flavus* NCIM 645, (6) *A. flavus* MTCC 152, (7) *A. parasiticus* and (8) *A. flavus* (Millet isolate)

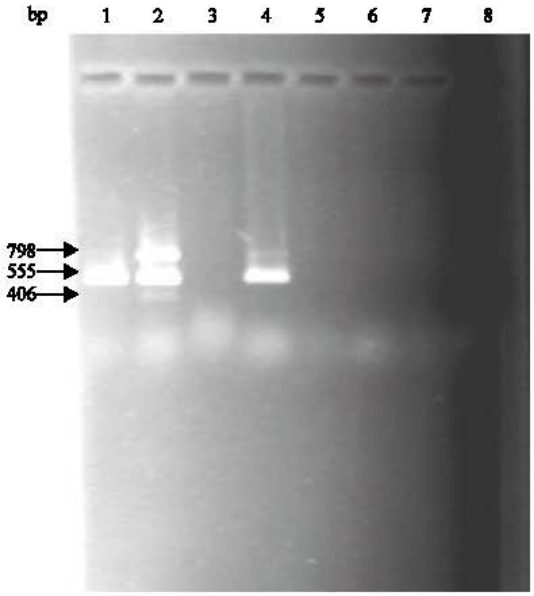

Fig. 2: PCR amplification of 18s rRNA region of different fungal species. Lanes (1) *A. ochraceus* CFR 221, (2) *A. flavus* (Millet), (3) *Fusarium* (Tomato isolate), (4) *A. oryzae* CFR 225, (5) *Fusarium* NCIM 665, (6) *Fusarium* NCIM 851, (7) *Fusairum* spp. and (8) Environmental Control

Table 3: Aflatoxin production by fungi

Fungal isolate	Toxin production (ppb)
A. flavus ATCC 46283	260
A. parasiticus CFR 223	200
A. flavus NCIM 645	180
A. flavus MTCC 152	160
A. flavus	160

primers. They obtained a four band pattern for all producers and a variable pattern for non-producers of aflatoxin. They argue that the lack of aflatoxin production apparently need not only be related to an incomplete pattern obtained in quadrauplex PCR. Different types of mutations may be responsible in inactivating the aflatoxin biosynthetic pathway genes in other *A. flavus* strains (Geisen, 1996). Yet, in another study (Liu and Chu, 1998), they studied quadruplex PCR using *avf*A, *omt*A, *ver*-1 and ITS primers. Their multiplex PCR assay gave positive results (tetrad banding pattern) for fermented foods. The ITS Universal primers anneal to the flanking Internal Transcribed Spacer (ITS) regions of the fungus 5.8s rDNA and amplify an approximately 600-bp amplicon and they are not specific for *Aspergilli*. Here only the aflatoxin biosynthetic pathway genes were used for the detection of aflatoxigenic *Aspergilli*, but *ITS* primer specific for the identification of genus *Aspergillus* was not used in their study.

In the present study, *aflR* and *omt* gene primers were used because *aflR* gene regulates the expression of *omt* gene, a structural gene enclosed in the aflatoxin biosynthetic pathway and *omt* gene is necessary for almost the final formalities of aflatoxin biosynthesis (Liu and Chu, 1998). The presence of these two genes clearly indicates the aflatoxin production machinery. With the use of *NS* primer pairs, *NS* indicate the presence of *Aspergillus* contamination in any commodity. The detection of aflatoxigenic strains with three bands corresponding to *aflR*, *omt* and *NS* genes confirm the identity of the invading genus.

Conventional methods using *Aspergillus* Differential Medium (ADM) however, could differentiate aflatoxin producers and non-producers. But this method is time consuming. Sometimes it may fail to identify the aflatoxin production because of instability of aflatoxin-producing strains growing on culture media (Abarca *et al*., 1988).

RT-PCR could be another biomolecular technique as a tool to differentiate aflatoxin-producing strains from non-aflatoxin producing strains of *A. flavus*, *A. parasiticus* and other *Aspergilli*, where in the presence or the absence of mRNA could help in direct differentiation between these strains. An RT-PCR assay using *tri*-5 gene provided a screening tool for trichothecene producing *Fusarium* species (Doohan *et al*., 1999).

PCR/RT-PCR are time-consuming and involve multiple steps thereby increasing the chances of contamination with exogenous DNA. The simultaneous identification of these agents in one step PCR procedure would offer a number of advantages over the conventional methods and would save both time and cost without compromising on the efficiency. Thus, the identification of a single toxigenic species is precise, simple, rapid and cost-effective detection assay which can be applied in food industry as an integral part of quality control programme for the assurance of raw products and clinical diagnosis. Thus, the multiplex PCR will be an even more powerful diagnostic tool for detecting aflatoxigenic *Aspergilli*.

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