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A MULTIPLEX PCR BASED METHOD FOR THE DETECTION OF AGRICULTURALLY IMPORTANT AFLATOXIGENIC AND OCHRATOXIGENIC *FUNGAL* SPECIES FROM CEREALS

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ABSTRACT

A total of 112 fungal isolates were recovered from cereal grains collected from 10 districts of Tamilnadu and Andhra Pradesh states. The collected samples were subjected to standard blotter method and different ochratoxigenic *Aspergillus* and *Penicillium* species were isolated and maintained. A multiplex PCR assay was standardized for simultaneous detection of toxigenic *Aspergillus* and ochratoxin producing *Penicillium* species targeting *pks1, otanps* and *AflR* genes involved in ochratoxin and aflatoxin (AflR) metabolic pathways respectively. An internal amplification control was incorporated into mPCR assay to eliminate the false-positive results during PCR reaction. To check the practical application of developed mPCR assay, a total of 112 cereal grain samples (56 maize and 56 paddy) were used. Out of 56 maize samples 17 had AFB1 positive, 23 had OTA contamination in detectable range. In the case of paddy 22 samples are positive for aflatoxin contamination and 13 were positive for ochratoxin contamination. Similar kind of findings was observed when the pure cultures of fungi were subjected to developed mPCR assay.

KEYWORDS: Aflatoxin, Ochratoxin, *Aspergillus, Penicillium*, Multiplex PCR, HPLC

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INTRODUCTION

Fungus contamination is one of the serious problems involving agricultural products, may occur at all stages of food production and storage and also it is very critical for the economics, food safety and human health perspectives. It has been reported that approximately 25 to 50 percent of world's agricultural crops are contaminated with mycotoxins.[1] Among the major mycotoxigenic fungi, genus *Aspergillus* is well known, widely spread and comprises 185 species, many of the species are opportunistic pathogens, liberate series of toxic secondary metabolites called *Aspergillus* toxins.[2] Toxins of *Aspergillus* species are mainly produced by certain strains of *Aspergillus flavus, A. paraciticus, A. niger, A. ochraceus, A. versicolar* and *A. nomius* when they grow on various agricultural crops such as ground nuts, maize, paddy, sorghum and variety of spices.[3,4] Till date, eighteen major *Aspergillus* toxins have been identified from different sources, however, only two of them have been found to be major contaminants in food and feed intended for human and animal consumption.[5]Among the major *Aspergillus* toxins, aflatoxins (AFB1, AFB2, AFG1, AFG2) and ocharatoxin A (OTA), stand more important in terms of their toxicity and impact. Aflatoxin B1 is the most toxic and classified as a group 1 carcinogen by the International agency for research on cancer.[6] These important *Aspergillus t*oxins can cause different types of cancers like liver cancer by AFB1, kidney cancers by OTA was well reported.[6,7] Extensive surveys conducted in different countries further revealed the presence of aflatoxins and OTA in food stuffs such as cereals, coffee beans, pulses, spices, meat and cheese products.[8,9] At a considerably high level of contamination in foods, these mycotoxins can cause toxic effects ranging from acute (liver or kidney deterioration), to chronic (mutagenic, teratogenic, carcinogenic) manifestations in humans and animals.[10] Over the past two decades, mycotoxicosis has been observed both in human and animals. Mycotoxicosis causes acute liver damage, liver cirrhosis, induction of tumors, attack on central nervous system, skin disorders and hormonal defects.[11] Due to their deleterious effects on humans and other farm animals, several countries implemented the regulations of mycotoxin limits in several food commodities intended for consumption. [12] India is largely being agriculture based economy that too in subtropical region needs to address this problem of mycotoxin assessment in crops and also need to establish regulatory limits. The conditions or mycotoxicosis caused by mycotoxins are not consistent; therefore, determining the cause of the specific condition or disease requires confirmation of the toxin(s) in a representative sample of the feed, food, tissue or fluid.[13] The level of fungal infection as well as the identification of main toxigenic fungal species is important not only for assessing food quality but also to develop control strategies for obtaining safe food.[14]

Surveillance for mycotoxins and mycotoxigenic fungi becomes critical for maintaining high quality grains and grain products in developing countries like India. The profile of mycotoxins present in commodities depends on both the environmental conditions and the toxigenic species present. Moreover, constant changes in the taxonomy of these genera may lead to misidentification of an isolate and false evaluation of its toxigenic potential. In fact many isolates may be not mycotoxigenic because of the influence of the environmental factors and gene mutations in one or more genes belonging to the toxin biosynthetic gene cluster.[15,16] To circumvent this problem, several researchers have used more specific and sensitive molecular tests like Enzyme Linked Immuno assays and Polymerase Chain reaction (PCR).[17] Both the approaches provide fast analysis of results but the PCR technology is preferable as more specific and sensitive for the early detection of toxigenic fungi.[18] Recently Niesson[19] reviewed PCR-based methods available for detection of

potential producers of aflatoxins and Ochratoxins. Certain food components, such as fats, polysaccharides, polyphenols, and other secondary compounds have been reported as major obstacles for an efficient amplification in PCR,[20] which leads to false positive results in PCR assays. To overcome those problems, incorporation of internal amplification control into diagnostic PCR's is mandate.[21]In present study *PKS* gene encoding polyketide synthase a key enzyme involved in ochratoxin metabolism specific to ochratoxigenic *Aspergillus* species were targeted, *AflR* gene encoding transcriptional regulation factor for aflatoxin biosyntahis in Aspergillus species was targeted for specific detection of aflatoxigenic *Aspergillus* species and also *otanps* gene encoding nonribiosomal peptide involved in ochratoxin metabolism in *Penicillium* species was targeted. The aim of the current study is to develop and evaluate a multiplex PCR assay for simultaneous detection of aflatoxin and ochratoxin producing fungal species from contaminated foods, with the application of an internal amplification control to eliminate the false positive results in PCR assay.

MATERIALS AND METHODS

(i) Sample collection

Infested cereal samples comprising maize, wheat and paddy were collected from high rainfall areas in Southern India such as Andhra Pradesh and Tamilnadu. A total of 112 samples (56 samples from each region) were collected during winter 2010-2012. All the samples were in uniform size of 250 grams of each was collected and stored at 4 ^{o}C for further analysis of aflatoxigenic and ochratoxigenic mycoflora and toxin detection.

(ii) Isolation and identification of moulds

The counts of fungal populations and dominant genera were isolated from the cereal samples. Grains were surface sterilized and placed onto PDA and the pure cultures were maintained in the Czapeck Dox agar medium. To identify the fungi at the genus level, the Manual of Barnett and Hunter[22] was used. The reference strains (Table-1) were obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India was used as a standard culture. Our morphological findings were further confirmed by species specific PCR assays.

(iii) Molecular detection of toxigenic fungi standardization of multiplex PCR assay

Extraction of DNA

DNA was extracted from pure cultures of fungi using DNeasy plant Minikit (Qiagen, Gambh, Germany, Cat-69106). A 10 ml of sterile potato dextrose broth (PDB) was inoculated with 500 µl of the single-spore culture suspensions and flasks were incubated under agitation (100 rpm) at room temperature for 3 days. The fungal cultures were then filtrated in a polyethylene membrane and washed twice with sterile distilled water. The membrane with the mycelium was folded and quickly dried with a tissue paper. The near dried fungal mycelium was transferred to an Eppendorf tube, frozen for 2 h at -20°C and lyophilized over night. Mycelial mat (100 mg) was ground in liquid nitrogen and total genomic DNA was extracted as per manufacturer's instructions. Nuclease free water was used to dilute the DNA stock solution and the concentration was estimated by using the Nanodrop (Thermo Scientific nanodrop 2000c) and DNA was stored at -80 ^{o}C until use.

Table 1
Standard cultures and their toxin chemotypes used in present study.

S.No	Strain name and Source	PCR			HPLC
		aflR	*pks*	*otanps*	Chemotype
1	*Aspergillus niger* ITCC *236*	Negative	Positive	Negative	OTA
2	*Aspergillus niger* ITCC 1436	Negative	Positive	Negative	OTA
3	*Aspergillus ochracious* ITCC 3167	Negative	Positive	Negative	OTA
4	*Aspergillus ochracious* ITCC 2454	Negative	Positive	Negative	OTA
5	*Aspergillus ochracious* ITCC 1810	Negative	Positive	Negative	OTA
6	*Penicillium verrucosum* ITCC 2156	Negative	Negative	Positive	OTA
7	*Penicillium verrucosum* ITCC 2986	Negative	Negative	Positive	OTA
8	*Penicillium verrucosum* MTCC 1758	Negative	Negative	Positive	OTA
9	*Penicillium veridicatum* MTCC 2007	Negative	Negative	Positive	OTA
10	*Aspergillus flavus ATCC 46283*	Positive	Negative	Negative	AFB1
11	*Aspergillus flavus NCIM 152*	Positive	Negative	Negative	AFB1
12	*Aspergillus flavus NCIM 645*	Positive	Negative	Negative	AFB1
13	*Aspergillus flavus NCIM 650*	Positive	Negative	Negative	AFB1
14	*Aspergillus flavus MTCC 2798*	Positive	Negative	Negative	AFB1
15	*Aspergillus parasiticus MTCC 2797*	Positive	Negative	Negative	AFB1
16	*Fusarium graminearum MTCC 2089*	Negative	Negative	Negative	Negative
17	*Fusarium verticelloides MTCC 3693*	Negative	Negative	Negative	Negative
18	*Fusarium sporotrichoides MTCC 2081*	Negative	Negative	Negative	Negative
19	*Penicillium chrysogenum MTCC 6479*	Negative	Negative	Negative	Negative
20	*Fusarium moniliforme MTCC 156*	Negative	Negative	Negative	Negative

Note: MTCC-Microbial Type Culture Collection; ITCC-Indian Type Culture Collection; ATCC- American Type Culture Collection; NCIM- National Collection of Industrial Microorganisms.

Isolation of DNA from contaminated food grains

Twenty grams of the contaminated food sample were ground in coffee grinder for 90s and then 0.2 grams ground grain was mixed in 1ml lysis buffer- (100 mM tris HCl, 50 mM EDTA, 150 mM NaCl and 1% SDS) and homogenized by gentle mixing and kept it in a water bath at 60 °C for 10 min. Samples were centrifuged at 12000 x *g* for 5 min and 500 µl of supernatant was mixed with 150 µl of 5 M potassium acetate and incubated on ice for 10 min. After centrifugation at 400 µl of supernatant was mixed with 300 µl of ice cold isopropanol to precipitate the DNA, the resulting pellet was washed with 70 % ethanol, dried under air and dissolved in 50 µl of Tris-EDTA (pH 8.0).

Primer designing

DNA sequences were analysed and aligned by Clustal method. Primers were designed using the aligned gene bank database sequences viz., *pks, otanps* and *AflR* primers were used for the specific detection of ochratoxigenic and aflatoxigenic (*AflR*) producing *Aspergillus* and *Penicillium* species respectively. Primers were designed using Gene runner software (http://www.generunner.com). Primer sequences are listed in Table.2. Before standardizing mPCR protocol, all primers were evaluated on to array of fungal species to check the specificity and sensitivity.

Designing of Internal Amplification Control

An IAC (internal amplification control) was constructed by targeting pUC19 DNA with 5' overhanging ends of aflr primer pair. The PCR reaction mixture for generation of IAC-DNA contained 500 nM of each primer, 200 µM of each dNTP (Sigma, India), 0.5 units of *Taq* DNA polymerase (Sigma, India), 2. 0 mM $MgCl_2$ in 1X PCR buffer (Sigma, India) and 250 pg of template DNA. PCR was carried out through 30 cycles in Eppendorf mastercycler gradient thermal cycler (Eppendorf Hamburg, Germany) under following reaction conditions: denaturation at 94 °C for 1 min, primer annealing at 58°C for 1 min and extension at 72 °C for 1.30 min in each cycle. The DNA was denatured initially for 4 min in the beginning and extended at 72 °C for 8 min. The PCR product was purified using the sPCR purification kit (Qiagen). The concentration of IAC-DNA was determined using a NanoDrop-ND- 1000 spectrophotometer and was stored at -20 ^{o}C. The following equation was used to calculate

the copy number of the PCR product concentration: weight of the PCR fragment (in g μl^{-1}) × (6.023 × 1023)/ (660 g mol^{-1} × number of base pairs of PCR fragment) = the number of genomic copies per microlitre.[21]

Multiplex PCR assay

Multiplex PCR was carried out for the detection of aflatoxin and ochratoxin producing *Aspergillus* and *Penicillium* sp., detection, in an Eppendorf master cycler gradient (Hamburg, Germany) with a reaction volume of 30 µl. The amplification mixture consisted of template DNA (1.0 µl), $MgCl_2$ (2.0 mM), 1X PCR buffer (Sigma, India), dNTP mix (200 µM, Sigma, India), *Taq* DNA polymerase (1 unit, Sigma, India) and primer pairs specific to the targeted genes *pks, otanps* and *AflR* were added at a concentration of 100 nM, 150 nM, 200 nM and 50 nM, respectively. IAC was added at a concentration of 1000 copies per reaction. The PCR cycling conditions were carried out with an initial denaturation at 94 °C for 4 min, followed by 30 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1.5 min, with a final extension of 72 °C for 8 min.

Specificity and sensitivity of mPCR on artificially contaminated maize grains

The specificity of the mPCR primers was determined against different organisms shown in (Table.2). Sterile maize grains (5 grams) were experimentally spiked with known toxigenic *Aspergillus* and *Penicillium* spore suspensions at different concentrations ($1x10^5$, $1x10^4$, $1x10^3$ and $1x10^2$ CFU g^{-1}). Negative controls were kept without inoculation of spores. All the samples were enriched for two days, further DNA was isolated and analysed by mPCR assay.

(iv) Profiling of toxin chemotypes

Extraction and cleanup of Aflatoxin and ochrotoxin A

The extraction of Aflatoxin and ochratoxin A was carried using immuno affinity columns. Briefly, 50 g of well ground sample / 100 ml of culture supernatant was extracted with 250 ml of acetonitrile–water (60:40, volume/volume) using high speed blending for 2 min. The extract was filtered through Whatman No. 4 filter paper and an equal volume of chloroform was added to the filtrate. Further cleanup was preceded with the chloroform fraction by the addition of PBS. The filtrates were passed through AflaCLEAN and otaCLEAN immuno affinity columns, (LC-TECH, USA) for recovery of aflatoxin and ochratoxin A respectively. These elutes were used for further HPLC analysis.

Reverse phase HPLC

Chemical detection of aflatoxins and ochratoxin A (OTA) was performed by HPLC (JASCO) equipped with a PDA detector. The separation was performed on RP - C18 column (250 mm x 46 mm) at a flow rate of 1ml/min and the injection volume was 20 µl, with water: methanol: acetonitrile (50:40:10 volume/volume) and methanol: acetonitrile: acetic acid (50:40:10 volume/volume) as mobile phase for AFB1 and OTA . Absorbance maximum was observed at 365 nm and 336 nm for AFB1 and OTA, respectively. The concentrations of AFB1 and OTA were calculated using a calibration curve of standard separately.

RESULTS AND DISCUSSIONS

In recent years, a growing number of PCR assays have been developed for surveillance of phyto-pathogenic microorganisms including mycotoxigenic fungi from agricultural, industrial (Food), medical and environmental samples.[23,24] Conventional methods for the detection of mycotoxigenic fungi based on sporodochia with abundant macroconidia on the chaff surface is time consuming and laborious; however, PCR assays have proven to be very useful and sensitive where sporodochia are absent or are poorly developed. It would be more meaningful if analytical systems were available at low cost, were simple to use, and could be used for qualitative and quantitative assessments of the mycotoxins present in different food matrices. The assay described in this research relies on 3 pairs of primers that amplify aflatoxgenic *Aspergillus*, ochratoxigenic

Aspergillus and *Penicillium* species under optimized PCR conditions. The genes targeted were *AflR* (400 bp size encoding transcriptional regulation factor for aflatoxin producing fungi, *pks* gene (650 bp size encoding *polyketide synthase*) for ochratoxigenic *Aspergillus* species, *otanps* (788 bp) encoding non ribosomal peptide synthase for ochratoxigenic *Penicillium* species detection. The assay fits well in to an analytical scheme that starts with the detection of the potential mycotoxigenic *Aspergillus* and *Penicillium* species.

1. Standardization of mPCR assay

The primer concentrations for mPCR were optimized for the respective target genes before standardization of mPCR assay. The mPCR was then optimized with an annealing temperature of 56 ° C and 1.5 mM $MgCl_2$ concentration. The concentrations of all the oligonucleotide primers targeting various genes and IAC DNA, were further adjusted in such a manner to provide equal intensities of all the amplified products. The standardized mPCR revealed the presence of 4 bands of nearly uniform intensity after agarose gel electrophoresis (Fig. 1). The genes targeted and the final primer concentrations employed and their amplicon sizes are depicted in Table 2. Special features of this assay are the incorporation of the internal amplification control to negotiate the false negative results during PCR assay and simultaneous detection of 3 major mycotoxin chemotypes usually found in cereals. In our previous study we reported an assay for the identification of mycotoxigenic *Fusarium* species.[15] However, the present assay has a broad range for the identification of other mycotoxigenic fungi, and hence it could be useful as a diagnostic tool in routine food analysis laboratories.

Figure 1
mPCR photograph

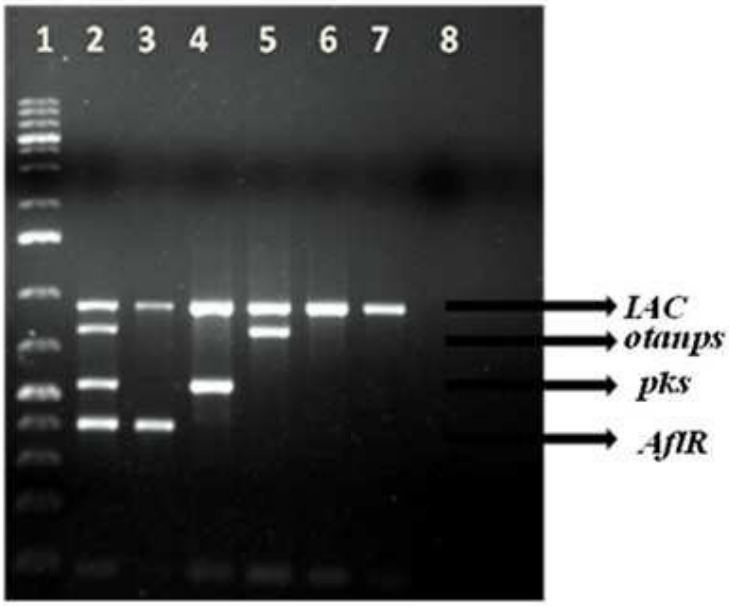


Figure1-mPCR standardization: Lane1-1 kb DNA ladder; Lane 2-Standardization of target gene amplification including IAC; Lane 3- sample contaminated with aflatoxigenic fungi; Lane 4- sample contaminated with ochratoxigenic Aspergillus species; Lane 5-sample contaminated with ochratoxigenic Penicillium species.

Table 2
Premiers used in this study and targeted gene amplicon sizes

Primer	Sequence (5'-3')	Target gene	Amplicon size
aflRF	GCACCCTGTCTTCCCTAACA	*aflR*	400 bp
aflR R	ACGACCATGCTCAGCAAGTA		
pksF	GGTCTATGCTCTGCGGTTCA	*Pks*	650 bp
pksR	TCAAAGGAGTAAGGCGGA		
Npspn F	GTGCGTGATCCTACCAGACA	*otanps*	788 bp
Npspn R	TTGGTGTCATGGCGAAAGGT		
IAC F	GCACCCTGTCTTCCCTAACA TAGACTGGATGGAGGCGG	Puc19DNA	988 bp
IAC R	ACGACCATGCTCAGCAAGTA AGTCAGAGGTGGCGAA		

2. Evaluation of mPCR assay on contaminated food grains (Sensitivity, specificity and detection limits)

The specificity of all the primers was assessed by performing mPCR on the genomic DNA of other standard strains listed in Table 1. No spurious products were observed when the DNA from non-specific organisms were used. In the present study a competitive internal amplification control was incorporated in mPCR assay to negotiating the false positive results during mPCR reaction and in order to make the mPCR assay acceptable to the present norms of diagnostic tool.[21] The IAC gene amplified under all conditions, demonstrating that the PCR mix and conditions were satisfactory. To determine the sensitivity, the developed mPCR was evaluated on to naturally and artificially contaminated food samples with a minimal enrichment time of 48 hrs. Enrichment of the food samples was required, since moulds are primarily found as spores or dried mycelia on dry food, and these are resistant to cellular disruption for DNA extraction.[25] Sensitivity of mPCR was standardized as 80 ng of purified genomic DNA of all the standard fungal species tested in the present study. When spore suspension dilutions were used, the sensitivity of the mPCR was 10^3 spores per ml for all the genes were targeted in the present study.

3. Application of mPCR on to pure cultures of Fungi

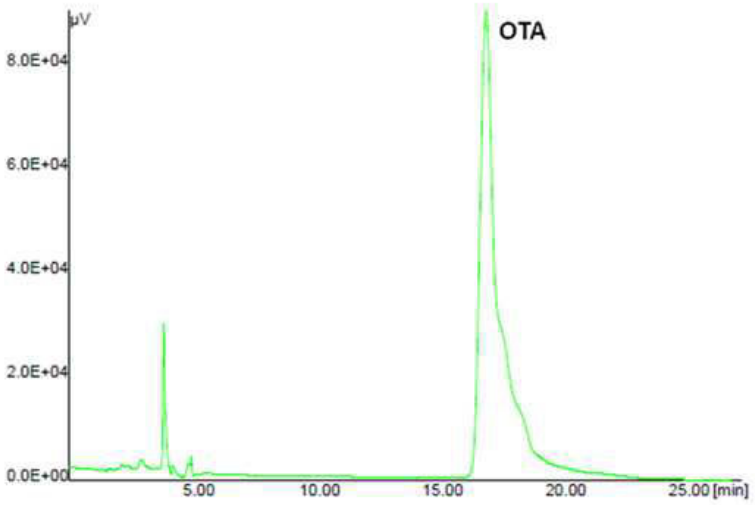
Primers for targeting genes were used to predict whether these isolates could produce mycotoxins (ochratoxin/aflatoxin)), out of 98 total isolates were recovered from food grain samples, 76 isolates were positive for toxin production by mPCR. Mycotoxin production was confirmed *in-vitro* by high pressure liquid chromatography (HPLC) in all mPCR positive isolates after growth in a liquid medium (Czapek-dox medium). Among the PCR positive ochratoxigenic and aflatoxigenic *Aspergillus* isolates 57 % and 62 % were scored positive for toxin production, in the case of *Penicillium* species 63 % was positive for toxin production *in-vitro*, rest (15%) were negative for toxin production. This kind of observations are not new since, Ramana et al., [15] were observed similar kind of findings. This could be reason that in field conditions many factors can influence the toxin production. In some cases, though, the toxin metabolic pathway genes are present, the ability to produce a particular mycotoxin is found lacking and these isolates may result in false positives in a PCR assay.

4. Application of multiplex PCR assay on to naturally contaminated samples

A total of 112 contaminated grains including maize (56) and paddy (56) collected from contaminated fields of Andhra Pradesh and Tamilnadu, India was subjected to the mPCR assay as well as HPLC analysis. No grain sample that was determined to be negative by mPCR assay had any mycotoxin in the sample. Out of 56 maize samples 17 had AFB1 positive, 23 had OTA contamination in detectable range by HPLC and rest were not observed with significant quantities of toxins. In the case of paddy 22 samples are positive for aflatoxin contamination and 13 were positive for ochratoxin contamination and the rest were

stayed negative by HPLC analysis. Fig-2 depict the HPLC chromatogram of standard OTA. The minor discrepancy observed might be the result of many factors that can influence toxin production. Sugar, salt, amino acid levels and moisture content in the production media might also affect toxin production.[26] Or, the intricacies involved in toxin extraction protocol or clean-up columns also would have resulted in a poor yield in mycotoxins.

Figure 2
HPLC chromatogram of standard Ochratoxion A



However, considering the economic importance of these mycotoxins and the pathogenicity of the fungi producing the mycotoxins, a fast and rapid mPCR assay, despite occasional false positives, would still be useful for the high-throughput analysis of fungal isolates because of its importance in real time scenario of biothreat perception towards humans and other farm animals due to mycotoxin contamination..

CONCLUSIONS

In conclusion the developed system provides an accurate, rapid, sensitive and cost effective detection of mycotoxigenic *Aspergillus* and *Penicillium* species in pure cultures as well as in the infected food grains. Also the developed system can able to avoid false positive results during PCR reaction. These tests will help to analyze the industrial food samples, food grains, and their products. The study will help to predict the mycotoxin accumulation during storage and transportation as well.

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