

A Novel Non Radioactive PCR-DNA Probe for the Detection of Aflatoxin Producing *Aspergillus* Species from Major Food Crops Grown in India*

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ABSTRACT

In the present study, a novel non radioactive digoxigenen labelled PCR-DNA probe was developed targeting *nor-1* gene to assess the contamination of aflatoxigenic *Aspergillus* species in food grain samples from Southern parts of India. The sensitivity of developed PCR-DNA probe was determined to be 10 pg of genomic DNA and 1 pg of purified PCR product. The specificity of the DNA probe was validated by testing against an array of *Aspergillus*, *Fusarium* and *Penicillium* spp. A total of 89 *Aspergillus* isolates were recovered from 152 grain samples of maize, paddy, and groundnut. Among them, maize had the highest (90%) incidence of toxigenic *Aspergillus* species. When developed PCR-DNA probe was evaluated onto pure cultures of toxigenic and nontoxigenic *Aspergillus* species, 60 samples were positive for the *nor-1* gene probe. DNA probe results unequivocally matched with the HPLC analysis. In conclusion, the novel PCR-DNA probe developed in this study may find its application in rapid detection of aflatoxin-producing *Aspergillus* isolates from contaminated cereal grains.

Keywords: AFB1-Aflatoxin B1; HPLC-High Performance Liquid Chromatography; *Nor-1*; Norsolorinic Acid Synthase; PCR-Polymerase Chain Reaction

1. Introduction

Aflatoxins are common mycotoxins in agriculture and food commodities causing serious health hazards to humans and animals leading to great economic losses. Among the aflatoxin group produced by *Aspergillus* species, Aflatoxin B1 is most potent carcinogen and it was classified as group 1 carcinogen by the International Agency for Research on cancer [1]. Although aflatoxin-production ability has been detected in various species of the *Aspergillus* genus, inside and outside the *Flavi* group, *Aspergillus flavus* and *Aspergillus parasiticus* remain the most important and representative aflatoxin producers occurring naturally in food commodities [2]. Several mycotoxicoses cases in humans and animals have been reported due to the consumption of mycotoxin contaminated food and feed [3].

Because of the toxic and potent carcinogenic properties of aflatoxins, many developed countries have established very stringent regulations limiting the maximum allowable amount of aflatoxins in food and feed [4]. Classical mycological analysis based on morphological and biochemical characters is time-consuming and not

always straight-forward. Molecular techniques have also been widely applied for distinguishing aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* by targeting one or several genes involved in the aflatoxin biosynthetic pathway with the ability/inability to produce aflatoxins. Polymerase Chain Reaction (PCR) methods have been developed for detection of aflatoxigenic fungi. In this respect, several primers have been designed for several genes in the biosynthetic pathways of aflatoxins; for example, *afl*, *nor*, *omt*, *ord*, *tub*, *ver* [5-9]. It has been demonstrated that most of the 25 identified genes clustered within a specific 70-kb region of the fungal genome are involved in aflatoxin biosynthesis [10]. Among them, *nor-1* gene encodes an enzyme that catalyses the ketoreduction of norsolorinic acid (the first stable pathway intermediate) to averantin, plays a major role in aflatoxin biosynthesis [11]. A large number of probe-based methods have already been developed for the detection of various food borne bacterial pathogens and mycotoxigenic fungi [12,13]. In the present study, a novel non-radioactive digoxigenen labelled PCR-DNA probe was developed targeting *nor-1* gene to assess the contamination of aflatoxigenic *Aspergillus* species in contaminated food grain samples. To know the incidence of toxigenic *Aspergillus* species and aflatoxin concentrations, a sur-

* Authors do not having any conflict of interest in present study.

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vey was conducted among the cereal grain growing regions in Southern India. Developed PCR-DNA probe was evaluated on to an array of fungal species recovered from contaminated food grains. For practical usefulness, the developed PCR-DNA probe was co evaluated with the chemical analysis of aflatoxins by HPLC.

2. Material and Methods

2.1. Fungal Strains, Media and Culture Conditions

The list of Standard strains used in the present study was given in (Table 1). Standard fungal strains were obtained from culture collection centers at the Microbial Type Culture Collection (MTCC), Chandigarh, the National Collection of Industrial Microorganisms (NCIM), Pune and the Indian Type culture Collection (ITCC), New Delhi, India. *Aspergillus* isolates were recovered from infected food grains (maize, paddy and groundnut) collected from different regions of Southern India including Andhra Pradesh, Kerela, Karnataka and Tamilnadu. A total of 62 maize, 50 paddy and 40 groundnut samples were subjected to standard blotter method (International Seed Testing Association, 1996) to assess the incidence of *Aspergillus* species and were sub cultured on to potato dextrose agar (PDA; Himedia, Mumbai, India) for 7 days at 25°C and single spore cultures were lyophilized and maintained at 4°C.

2.2. Molecular Detection of *Aspergillus* Species DNA Extraction

The DNA isolation from standard strains and grains was

carried out with minor modifications of Ramana *et al.* [14]. The DNA was dissolved in 50 µL of 2 mM Tris-EDTA and stored at -20°C. DNA concentrations were determined using a NanoDrop-ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA).

2.3. Species Specific PCR Assays: Primers and Polymerase Chain Reaction (PCR) Conditions

Species specific PCR assays were carried out by using reported primers PEPO1, PEPO2 and AnF, AnR were used for specific detection of *A. flavus* and *A. niger* respectively [28,29]. Primer sequence and references were given in (Table 2).

2.4. Design of *Nor-1* Gene Specific Oligonucleotide Primers and PCR Assay

One set of oligonucleotide primers were designed targeting *nor-1* gene involved in aflatoxin biosynthesis for developing PCR-DNA probe. The *nor-1* gene sequence was obtained from NCBI database (accession No. AY-510451.1). Gene runner software, version 3.1

(www.generunner.com) was used to design optimal primers: NOR1 F

5'ACCGCTACGCCGGCGCTCTCGGCAC 3' and NOR 1 R 5'GTTGGCCGCCAGCTTCGACACTCCG 3'. PCR amplification was performed in a 20 µL reaction volume with 0.5 µM each forward and reverse primers, 2 µL of 10 × PCR buffer, 1.5 mM of MgCl₂, 80 µM of each dNTP's, and 1 U of *Taq* polymerase (MBI Fermentas) in Eppendorf master thermal cycler (Hamburg, Germany).

Table 1. Standard isolates used in this study.

S No.	Strain name and source	DNA probe result	HPLC result
1	<i>Aspergillus flavus</i> ATCC 46283	+	+
2	<i>Aspergillus flavus</i> NCIM 152	+	+
3	<i>Aspergillus flavus</i> NCIM 645	+	+
4	<i>Aspergillus flavus</i> NCIM 650	+	+
5	<i>Aspergillus flavus</i> MTCC 2798	+	+
6	<i>Aspergillus Parasiticus</i> MTCC 2797	+	+
7	<i>Aspergillus Parasiticus</i> MTCC 2796	+	+
8	<i>Aspergillus parasiticus</i> NCIM 898	+	+
9	<i>Aspergillus niger</i> MTCC 9687	-	-
10	<i>Fusarium moniliforme</i> MTCC 156	-	-
11	<i>Fusarium sporotrichoides</i> MTCC 2081	-	-
12	<i>Fusarium graminearum</i> MTCC 2089	-	-
13	<i>Fusarium verticelloides</i> MTCC 3693	-	-
14	<i>Penicillium chrysogenum</i> MTCC 6479	-	-

Table 2. Primers used in this study.

Sl. No	Primer name	Sequence (5'-3')	Targeted gene	TM	Target organism	Size (bp)	References
1	PEPO1	CGACGTCTACAAGCCTTCTGGAAA	Aspergillopep-sin 1st exon	56°C	<i>A. flavus</i>	200	Lagotheti <i>et al.</i> , 2009 [28]
	PEPO2	CAGCAGACCGTCATTGTTCTTGTC					
2	ANF	CAGTCGTCCAGTACCCCTAAC	RAPD marker	56°C	<i>A. niger</i>	372	Sartori <i>et al.</i> , 2006 [29]
	ANR	GAGCGAGGCTGATCTAAGT					

A standard protocol was used to perform *nor-1* gene PCR. PCR program consisted of an initial denaturation of 4 min at 94°C followed by 30 cycles denaturation at 94°C for 40 sec, annealing at 58°C for 1 min and extension at 72°C for 90 seconds. A final extension of 72°C for 5 min was given at the end of the PCR reaction. The amplified product was examined by 1% agarose gel electrophoresis under UV.

2.5. DNA Probe Preparation

The DNA probe was synthesized by PCR amplification of *nor-1* gene from the *A. flavus*. The PCR amplicon was purified using the QIAQuick PCR purification kit (Qiagen, Germany) and labeled by randomly priming with alkali label Digoxigenin-11-dUTP nucleotide following the manufacturer's instructions (Roche Diagnostics, India). Briefly, a 15 µL volume of purified *nor-1* gene PCR amplicon was boiled for 10 min and immediately chilled in ice to obtain completely denatured strands. Two microliters of 10 × hexanucleotide mixes, 2 µL of dNTP labeling mix and 1 µL of klenow enzyme labeling grade, were mixed with the above prepared PCR product and incubated overnight at 37°C. The reaction was stopped by heating at 65°C for 10 min.

2.6. Blotting and DNA Hybridization

The extracted DNA of *Aspergillus* and other fungal strains was boiled with the addition of 0.4 M NaOH and 10 mM EDTA in a volume of 30 µL for 10 min followed by instant cooling on ice to obtain single stranded DNA. These DNA mixtures were neutralized by adding an equal volume of 2 M ammonium acetate and blotted onto positively charged Hybond N⁺ nylon membrane soaked in 6 × SSC using biodot vacuum manifolds (Biorad laboratories, USA). Cross linking of denatured genomic DNA was carried out using UV cross-linker (UVP). The Dot blots were pre incubated in dig easy Hyb solution with shaking for 30 min at 42°C. Hybridization was carried out in dig easy hybridization solution (10 mL) with 50 ng/mL of dig-labeled DNA probe at 42°C in rotating glass tubes, overnight, after overnight hybridization, stringent washes were performed. The membrane was washed twice for 5 min in 50 mL 2 × SSC, 0.1% SDS at ambient temperature (25°C) under constant agitation. The

membranes were then washed in 50 mL 0.5 × SSC, 0.1% SDS (pre-warmed to wash temperature) at 58°C under constant agitation. After hybridization and stringent washes, the membrane was rinsed briefly (1 - 5 min) in 50 mL washing buffer and incubated for 30 min in 50 mL blocking solution then incubated for another 30 min in 50 mL antibody solution. After incubation membrane was washed twice for 15 min in 50 mL washing buffer and then equilibrated for 2 - 5 min in 50 mL detection buffer. The membrane was incubated in 50 mL freshly prepared colour substrate solution in a dark colored container with a lid in dark cabinet/room. During colour development the container was not shaken. The membranes were exposed to light for short time periods for colour development monitoring. The reaction was stopped when the desired band intensities were achieved, by washing the membrane for 5 min with distilled water and further documented.

2.7. Specificity and Sensitivity of PCR-DNA Probe

The sensitivity of PCR-DNA probe was determined directly onto the DNA prepared from genomic DNA extracted from *A. flavus* ATCC 46283. 10 ng of DNA was serially diluted and each dilution was analyzed by the optimized DNA hybridization assay. Also sensitivity of the PCR-DNA probe was determined against purified PCR amplicon of *nor-1* gene. The highest sensitivity in both the experiments was determined as the lowest concentration of genomic DNA/*nor-1* amplicon required to give a visible chromogenic signal on nylon membrane. At the same time, spore suspensions of the organism serially diluted in sterile saline was spiked on to the sterile maize grains. After 48 hrs of incubation DNA was extracted from the maize grains and subjected to dot blot hybridization.

2.8. Chemical Analysis of Aflatoxins by HPLC: Extraction and Clean-Up of Aflatoxins from Food Grains

The extraction of aflatoxins was carried out as per the procedure of Stroka *et al.* [15]. Immune affinity columns AflACLEAN (India) was used to clean up the aflatoxins as per the manufacturer instructions. These elutes were

used for HPLC analysis. HPLC was carried out by the method described by Ferreira *et al.* 2004 [16].

3. Results

3.1. Molecular Detection of *Aspergillus* Species

A total of 152 seed samples of maize, paddy and ground nut collected from different parts of Southern India were screened to recover *Aspergillus* species. Four different species of *Aspergillus* were recovered *A. flavus* (51), *A. niger* (15), *A. oryzae* (21) and *A. fumigatus* (2) (**Figure 1**). DNA from all *A. flavus* and *A. niger* isolates were isolated and subjected to PCR using the species specific PCR assays. When primer pair PEPO1 and PEPO2 was used all the *A. flavus* strains were given single fragment

of about 200 bp amplicon (**Figure 2(a)**), in the case of *A. niger*, 372 bp amplicon was obtained with the primer set AnF and AnR (**Figure 2(b)**). Amplification was not observed when these PCR assays were employed on other *Aspergillus* species viz., *A. oryzae*, *A. fumigatus*, *A. ochraceus* and *A. carbonarius*. *A. oryzae* and *A. fumigatus* were identified by monographs of Pitt *et al.* [17].

3.2. DNA Hybridization Analysis Using *Nor-1* Gene DNA Probe

DNA dot blot analysis using the *nor-1* gene probe was carried out with the fungal strains listed in (**Table 1**). The DNA probe hybridized with DNA extracted from standard toxigenic strains of *Aspergillus* isolates from con-

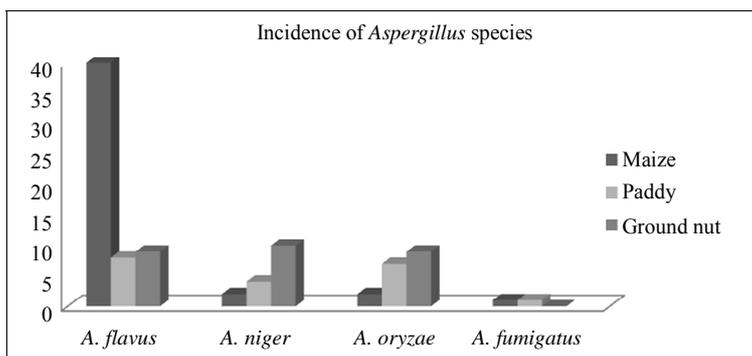
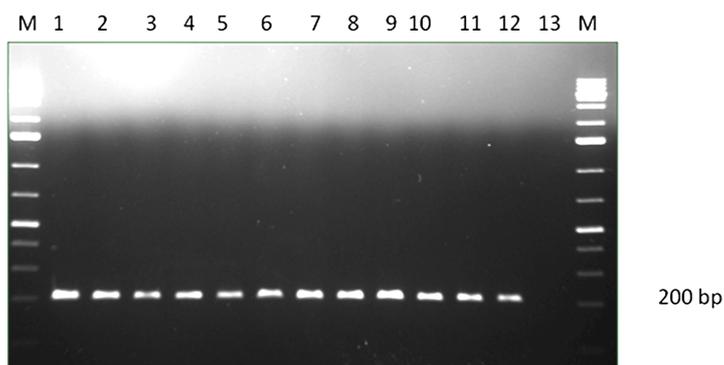
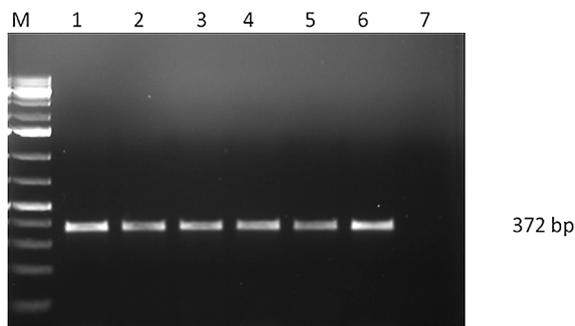


Figure 1. Incidence of *Aspergillus* species.



(a) Lane M-1: Kb DNA marker, lane 1-12: *A. flavus* strains, Lane 13: negative control.



(b) Lane M-1: Kb DNA marker, lane 1-6: *A. niger* strains, Lane 7: negative control.

Figure 2. (a) PCR amplification of *A. flavus*; (b) PCR amplification of *A. niger*.

taminated food grains exhibited positive chromogenic signal, whereas no hybridization signal was detected with other non toxigenic *Aspergillus* strains including *A. flavus* complex and other fungal strains included in this study (Figure 3). Among the 89 isolates of *Aspergillus* 60 strains were found to be positive by *nor-1* gene probe.

3.3. Detection Sensitivity

The sensitivity of developed PCR-DNA probe was evaluated to various dilutions of purified PCR product (ranging from 10 ng to 0.01 pg) was achieved as low as 0.01 pg (Figure 4). The detection limit of PCR-DNA probe was estimated onto the DNA purified by conventional protocol. The PCR-DNA probe could detect 10 pg genomic DNA. The sensitivity achieved by PCR-DNA probe was 1×10^6 CFU per gram of the seed sample. The reproducibility was checked by repeating the experiment twice.

3.4. Comparative Analysis of PCR-DNA Probe with HPLC

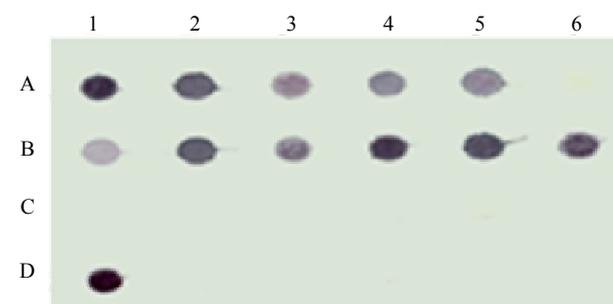
The extracted toxins were analyzed by HPLC along with $10 \mu\text{g}\cdot\text{mL}^{-1}$ of Aflatoxins standards (B1, B2, G1 and G2). The toxin potentials and quantification was determined by HPLC (Figure 5). When developed PCR-DNA probe was comparatively evaluated with conventional HPLC method, all aflatoxin positive genotypes of *Aspergillus* stayed positive for chemical analysis by HPLC (Table 3).

4. Discussion

In the present study a non-radioactive *nor-1* gene DNA probe was designed for specific detection and differentiation of aflatoxigenic and non-toxigenic *Aspergillus* species. The *nor-1* gene targeted for probe encodes a ketoreductase that regulates the major steps in aflatoxin metabolism [18]. Several experiments were conducted to prove the effective role of this gene in aflatoxin biosynthesis [19,20]. A large number of probe-based methods have already been developed for the detection and enumeration of various food borne bacterial pathogens and also for fumonisin producing *Fusarium* species [12,13,21,22]. In *Aspergillus* DNA probe was characterized for distinguishing strains of *A. flavus* [23], but so far toxigenic potential of the species has not been targeted.

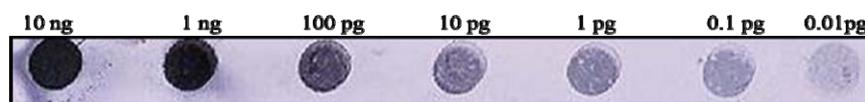
Molecular detection of aflatoxigenic *Aspergillus* species from food and clinical samples relies mainly on PCR amplification of specific target genes of interest. PCR methods have been found useful for detection of toxigenic *Aspergillus* present in contaminated food samples [24]. However, in PCR assays specificity mainly rely on 20 to 25 bases primer and also the stringent practical conditions that require expertise to standardize a reliable and robust PCR prior to evaluation. Dot-blot hybridization and reverse line hybridization assays are more attractive alternatives to overcome these problems. In the case of reverse line dot blot technique, the gene specific oligonucleotide probes are bound to the filter instead of the PCR product and DNA labeled during PCR amplification is used to hybridize to immobilized array of probes and only a single sample can be identified positively for different pathogens simultaneously. In contrast, a dot blot assay can analyze many samples for the presence of a single organism, as the purified DNA/PCR products from the samples are blotted on the membrane and hybridized to a single probe specific for the particular organism. In dot blot hybridization, the specificity is also more reliable as the targeted gene is checked on its entire probe length which would prevent the false positive results [25].

Among the cereal grains examined from Southern parts of India during 2010-2012. Maize had shown highest incidence of toxigenic *Aspergillus* species (90%) followed by groundnut (86%) and paddy (79%). The previous reports suggested that, maize is the most susceptible crop for aflatoxin contamination in tropical regions like



Lane A1to A5: aflatoxigenic *Aspergillus flavus* and standard strains of *Aspergillus flavus* and *Aspergillus parasiticus* strains; A6: negative control, Lane B1-B6 isolates of *Aspergillus*, C1-C6: standard strains of *Fusarium* and *Penicillium*, D1: Positive control, Lane D2-D6: other fungal species including non toxigenic *Aspergillus*.

Figure 3. Dot blot analysis of *nor-1* gene.



Sensitivity of PCR-DNA probe on serially diluted *nor-1* PCR amplicon ranging from 75 pg to 0.5 pg of DNA (1 pg was considered as detection limit).

Figure 4. Detection sensitivity.

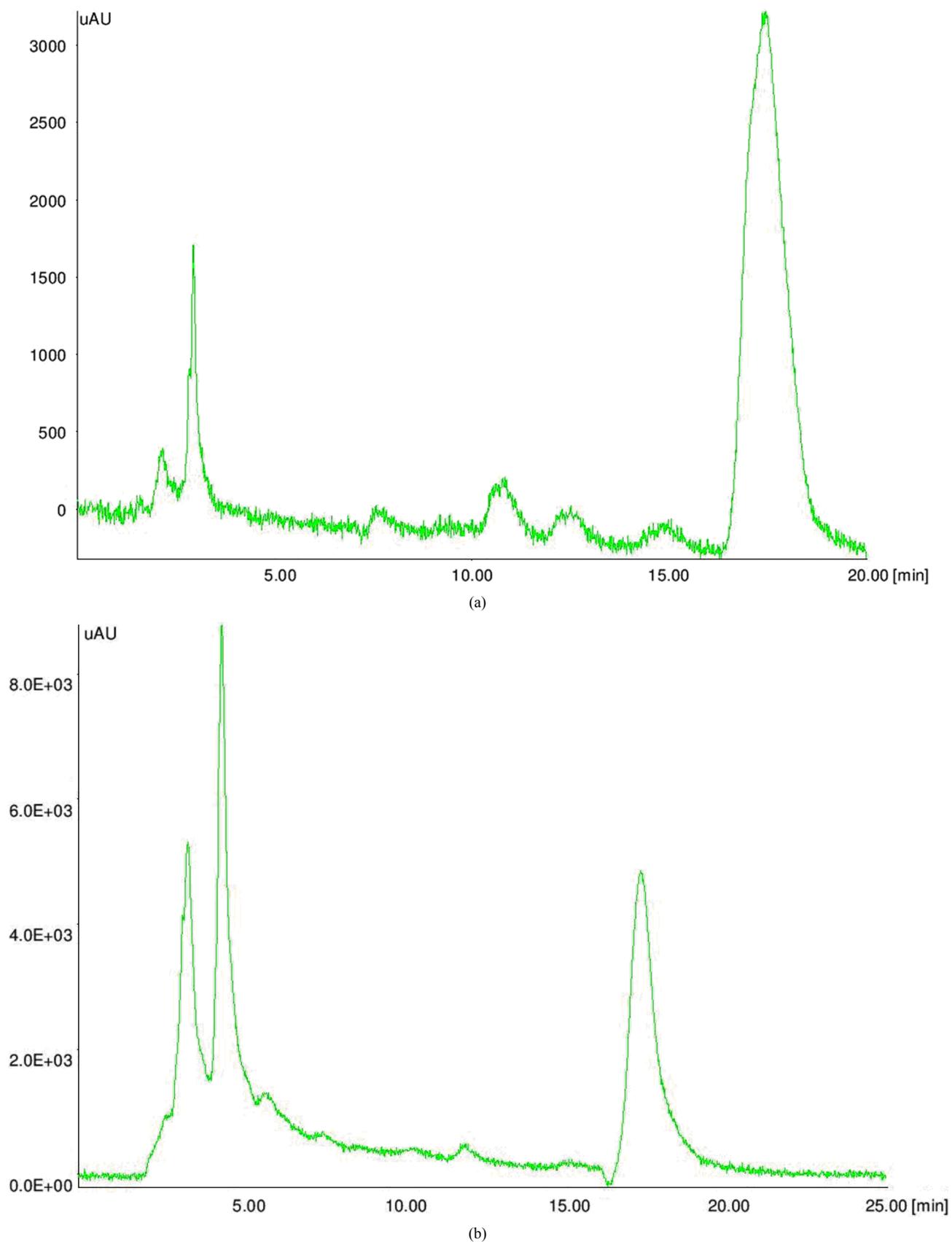


Figure 5. HPLC chromatograms. HPLC chromatogram of (a) aflatoxin standard B1 and (b) aflatoxin positive maize sample.

Table 3. Comparative evaluation of PCR-DNA probe with HPLC.

Sl.No.	sample	PCR-DNA PROBE	HPLC ($\mu\text{g/g}$)
1	KAM1	+VE	48.56802
2	KAM2	+VE	12.88403
3	KAM3	+VE	614.0154
4	KAM4	+VE	683.9237
5	KAM5	+VE	103.7235
6	KAM6	+VE	240.314
7	KAM7	+VE	85.59682
8	KAM8	+VE	135.3776
9	KAM9	+VE	125.9199
10	KAM10	+VE	130.094
11	KAM11	+VE	106.5748
12	KAM12	-VE	0
13	KAM13	+VE	145.1388
14	KAM14	+VE	28.76856
15	KAM15	+VE	32.46128
16	APM16	+VE	11.00326
17	APM17	+VE	0.234927
18	APM18	-VE	0
19	APM19	+VE	0.642744
20	APM20	+VE	145.1388
21	APM21	-VE	0
22	APM22	-VE	0
23	APM23	+VE	21.546
24	APM24	+VE	13.56544
25	APM25	+VE	34.675
26	APM26	-VE	0
27	TNM27	+VE	37.8732
28	TNM28	+VE	0.544
29	TNM29	+VE	87.657
30	TNM30	+VE	56.978
31	TNM31	+VE	32.867
32	TNM32	+VE	43.786
33	TNM33	+VE	0.768
34	TNM34	+VE	0.54
35	TNM35	+VE	1.786

Continued

36	TNM36	+VE	11.38735
37	TNM37	+VE	9.68
38	TNM38	+VE	45.765
39	TNM39	-VE	0
40	TNM40	-VE	0
41	TNM41	-VE	0
42	TNM42	+VE	5.69
43	KEM43	+VE	56.7833
44	KEM44	-VE	0
45	KEM45	-VE	0
46	KAP1	+VE	27.865
47	KAP2	+VE	10.650
48	KAP3	+VE	72.011
49	TNP4	-VE	0
50	TNP5	+VE	4.8256
51	TNP6	+VE	3.8843
52	TNP7	+VE	74.576
53	TNP8	+VE	29.321
54	TNP9	-VE	0
55	TNP10	+VE	82.665
56	KEP11	+VE	32.987
57	KEP12	+VE	12.563
58	KEP13	-VE	0
59	KEP14	+VE	1.654
60	KEP15	+VE	8.765
61	APP16	+VE	45.87
62	APP17	+VE	32.768
63	APP18	-VE	0
64	APP19	+VE	45.8743
65	APP20	+VE	7.865
66	APGN1	-VE	0
67	APGN2	+VE	38.6736
68	APGN3	+VE	13.6413
69	APGN4	+VE	38.216
70	APGN5	+VE	120.72
71	APGN6	+VE	23.50
72	APGN7	-VE	0

Continued

73	APGN8	+VE	96.7731
74	APGN9	+VE	9.4394
75	APGN10	+VE	110.333
76	APGN11	+VE	50.2311
77	APGN12	+VE	18.321
78	APGN13	+VE	4.674
79	APGN14	+VE	71.43
80	KAGN15	+VE	15.645
81	KAGN16	+VE	61.564
82	KAGN17	+VE	34.876
83	KAGN18	+VE	0.654
84	TNGN19	+VE	1.654
85	TNGN20	-VE	0
86	TNGN21	-VE	0
87	TNGN22	-VE	0
88	TNGN23	+VE	65.8743
89	TNGN24	+VE	18.56

India [26,27]. When developed PCR-DNA probe was evaluated on all the aflatoxin positive samples as well as fungal strains were showed positive for the *nor-1* gene. DNA-probe results unequivocally matched with the HPLC analysis.

In conclusion, the high levels of toxigenic strains found in many of the cereal samples warrants the need for better surveillance and monitoring by policy makers or food toxicologists to reduce the exposure of humans and animal life from aflatoxins produced by *Aspergillus* species. For this purpose, the designed DNA probe, besides having sensitivity and specificity equal to the molecular methods described earlier could score over others in terms of reliability, simplicity, ease of readability of results and safety in terms of avoiding carcinogenic chemicals.

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REFERENCES

- [1] IARC, "Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins," IARC Monographs on the Evaluation of Carcinogenic Risks to Human, International Agency for Research on Cancer, Lyon, Vol. 56, 1993.
- [2] J. W. Cary and K. C. Ehrlich, "Aflatoxigenicity in *Aspergillus*: Molecular Genetics, Phylogenetic Relationships and Evolutionary Implications," *Mycopathologia*, Vol. 162, No. 3, 2006, pp. 167-177.
- [3] M. Peraica and A. M. Domijan, "Mycotoxins in Food and Human Health," *Archives of Industrial Hygiene and Toxicology*, Vol. 52, No. 1, 2001, pp. 23-35.
- [4] T. J. Hansen, "Quantitative Testing for Mycotoxins," *American Association Cereal Chemists*, Vol. 38, 1993, pp. 346-348.
- [5] R. Geisen, "Multiplex Polymerase Chain Reaction for the Detection of Potential Aflatoxin and Sterigmatocystin Producing Fungi," *Systematic and Applied Microbiology*, Vol. 19, 1996, pp. 388-392.
[doi.10.1016/S0723-2020\(96\)80067-1](https://doi.org/10.1016/S0723-2020(96)80067-1)
- [6] G. Criseo, A. Bagnara and G. Bisignano, "Differentiation of Aflatoxin Producing and Non-Producing Strains of *Aspergillus flavus* Group," *Letters in Applied Microbiology*, Vol. 33, No. 4, 2001, pp. 291-295.
[doi.10.1046/j.1472-765X.2001.00998](https://doi.org/10.1046/j.1472-765X.2001.00998)
- [7] P. Rodrigues, A. Venancio, Z. Kozakiewicz and N. Lima, "A Polyphasic Approach to the Identification of Aflatoxigenic and Non-Aflatoxigenic Strains of *Aspergillus* Section Flavi Isolated from Portuguese Almonds," *International Journal of Food Microbiology*, Vol. 129, No. 2, 2009, pp. 187-193.
- [8] P. Rahimi, B. Sharifnabi and M. Bahar, "Detection of Aflatoxin in *Aspergillus* Species Isolated from Pistachio in Iran," *Journal of Phytopathology*, Vol. 156, No. 1, 2008, pp. 15-20.

- [9] B. Scherm, M. Palomba, D. Serra, A. Marcello and Q. Migheli, "Detection of Transcripts of the Aflatoxin Genes AflD, AflO, and AflP by Reverse Transcription-Polymerase Chain Reaction Allows Differentiation of Aflatoxin-Producing and Non-Producing Isolates of *Aspergillus Flavus* and *Aspergillus parasiticus*," *International Journal of Food Microbiology*, Vol. 98, No. 2, 2005, pp. 201-210.
- [10] C. A. Townsend, "Progress towards a Biosynthetic Rationale of the Aflatoxin Pathway," *Pure and Applied Chemistry*, Vol. 58, No. 2, 1997, pp. 227-238.
- [11] F. Trail, N. Mahanti, M. Rarick, M. Mehig, S. H. Liang, R. Zhou and J. E. Linz, "Physical and Transcriptional Map of an Aflatoxin Gene Cluster in *Aspergillus parasiticus* and Functional Disruption of a Gene Involved Early in the Aflatoxin Pathway," *Applied and Environmental Microbiology*, Vol. 61, No. 7, 1995, pp. 2665-2673.
- [12] A. R. Datta, B. A. Wentz, D. Shook and M. W. Trucksess, "Synthetic Oligodeoxyribonucleotide Probes for Detection of *Listeria monocytogenes*," *Applied and Environmental Microbiology*, Vol. 54, 1988, pp. 2933-2937.
- [13] M. V. Ramana, S. Chandrayanaka, K. Balakrishna, H. S. Murali and H. V. Batra, "A Novel PCR-DNA Probe for the Detection of Fumonisin Producing *Fusarium* Species from Major Food Crops Grown in Southern India" *Mycology: An International Journal on Fungal Biology*, Vol. 3, 2012, pp. 167-220.
- [14] M. V. Ramana, K. Balakrishna, H. S. Murali and H. V. Batra, "Multiplex PCR-Based Strategy to Detect Contamination with Mycotoxigenic *Fusarium* Species in Rice and Finger Millet Collected from Southern INDIA," *Journal of the Science of Food and Agriculture*, Vol. 91, No. 9, 2011, pp. 1666-1673.
- [15] J. Stroka, E. Anklam, U. Jorissen and J. Gilbert, "Immuno-Affinity Column Clean-Up with Liquid Chromatography Post Column Bromination for Determination of Aflatoxins in Peanut Butter, Pistachio Paste, Fig Paste and Paprika Powder: Collaborative Study," *Journal of Association of Analytical Communication and Interaction*, Vol. 83, No. 2, 2000, pp. 320-340.
- [16] I. Ferreira, E. Mendes and M. Oliveira, "Quantification of Aflatoxins B1, B2, G1, and G2 in Pepper by HPLC/Fluorescence," *Journal of Liquid Chromatography*, Vol. 27, No. 2, 2004, pp. 325-334.
- [17] J. I. Pitt, R. A. Samson and J. C. Frisvad "List of Accepted Species and Their Teleomorphs in the Family Trichocomaceae," In: R. A. Samson and J. I. Pitt, Eds., *Integration of Modern Taxonomic Methods for Penicillium and Aspergillus*, Harwood Academic Publishers, Amsterdam, 2000, pp. 9-47.
- [18] G. A. Payne and M. P. Brown, "Genetics and Physiology of Aflatoxin Biosynthesis," *Annual Review of Phytopathology*, Vol. 36, 1998, pp. 329-362.
- [19] J. Yu, J. W. Cary, D. Bhatnagar, T. E. Cleveland, N. P. Keller and F. S. Chu, "Cloning and Characterization of a cDNA from *Aspergillus parasiticus* Encoding an O-Methyl-Transferase Involved in Aflatoxin Biosynthesis," *Applied and Environmental Microbiology*, Vol. 59, No. 11, 1993, pp. 564-571.
- [20] F. Trail, N. Mahanti, M. Rarick, M. Mehig, S. H. Liang, R. Zhou and J. E. Linz, "Physical and Transcriptional Map of an Aflatoxin Gene Cluster in *Aspergillus parasiticus* and Functional Disruption of a Gene Involved Early in the Aflatoxin Pathway," *Applied and Environmental Microbiology*, Vol. 61, No. 7, 1995, pp. 2665-2673.
- [21] G. S. Sandhu, B. Kline, L. Stockman and G. D. Roberts, "Molecular Probes for Diagnosis of Fungal Infections," *Journal of Clinical Microbiology*, Vol. 33, No. 11, 1995, pp. 2913-2919.
- [22] J. L. Jones, "DNA Probes: Application in the Food Industry," *Trends in Food Science and Technology*, Vol. 2, 1991, pp. 28-32.
- [23] C. E. McAlpin and B. Mannarelli, "Construction and Characterization of a DNA Probe for Distinguishing Strains of *Aspergillus flavus*," *Applied and Environmental Microbiology*, Vol. 61, No. 3, 1995, pp. 1068-1072.
- [24] M. Z. Abdin, M. Malik and A. J. Saleem, "Advances in Molecular Detection of *Aspergillus*: An Update," *Archives of Microbiology*, Vol. 192, No. 6, 2010, pp. 409-425.
- [25] C. A. Lévesque, C. E. Harlton and A. W. A. M. De-Cock, "Identification of Some Oomycetes by Reverse Dotblot Hybridization," *Phytopathology*, Vol. 88, No. 9, 1998, pp. 213-222.
- [26] G. R. Janardhana, K. A. Raveesha and H. S. Shetty, "Mycotoxin Contamination of Maize Grains Grown in Karnataka (India)," *Food and Chemical Toxicology*, Vol. 37, No. 8, 1999, pp. 863-868.
[doi:10.1016/S0278-6915\(99\)00067-8](https://doi.org/10.1016/S0278-6915(99)00067-8)
- [27] S. V. Reddy, D. K. Mayi, M. U. Reddy, K. Thirumala-Devi and D. V. R. Reddy, "Aflatoxins B1 in Different Grades of Chilies (*Capsicum annum* L.) in India as Determined by Indirect Competitive ELISA," *Food Additives and Contaminants*, Vol. 18, No. 6, 2001, pp. 553-558.
- [28] M. Logotheti, A. Kotsovoli-Tseleni, G. Arsenis and N. I. Legakis, "Multiplex PCR for the Discrimination of *A. fumigatus*, *A. flavus*, *A. niger* and *A. terreus*," *Journal of Microbiological Methods*, Vol. 76, 2009, pp. 209-211.
- [29] D. Sartori, M. C. Furlaneto, M. K. Martins, M. R. F. Paula, A. Pizzirani-Kleiner, M. H. Taniwaki and M. H. P. Fungaro, "PCR Method for the Detection of Potential Ochratoxin-Producing *Aspergillus* Species in Coffee Beans," *Research in Microbiology*, Vol. 157, No. 4, 2006, pp. 350-354. doi.org/10.1016/j.resmic.2005.09.008