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Detection of aflatoxigenic fungi in selected food commodities by PCR

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Abstract

Aflatoxins are toxic secondary metabolites produced by *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*. A regulatory gene, *aflR*, is involved in regulation of aflatoxin biosynthesis and the sequence has been published. In this study, rapid assessment of aflatoxigenic fungi in food was accomplished using an indigenously designed primer pair for the aflatoxin regulatory gene *aflR* in polymerase chain reaction (PCR). Specificity was assayed in pure and mixed culture systems using DNA extracted from 28 different fungal strains as PCR template. Positive amplification was achieved only with DNA from aflatoxigenic *A. flavus* and *A. parasiticus*. The detection limit for mycelium and spores was determined as 0.05 g and ≥100 cfu, respectively. Specificity and sensitivity of PCR assay in groundnuts and maize for *A. flavus* was possible with as few as 100 cfu/g.

Keywords: Aflatoxins; *Aspergillus flavus*; *Aspergillus parasiticus*; PCR; *aflR*; Food commodities

1. Introduction

Aflatoxins are secondary metabolites produced by the aflatoxigenic fungi *Aspergillus flavus* Link and *Aspergillus parasiticus* Speare [1]. Aflatoxin contamination of agricultural commodities had gained global significance as a result of their deleterious effects on human and animal health as well as their importance to international trade. The contamination of foods by aflatoxigenic fungi, especially in tropical countries may occur during preharvesting, processing, transportation and storage [2]. These fungi mainly infect maize, cotton, peanuts, treenuts [3], figs [4] and spices [5–7].

One of the most important requirements for eliminating aflatoxin is the identification of mycotoxigenic fungal contamination. The detection of the aflatoxigenic fungi is usually performed by traditional dilution plating, use of diagnostic media or by immunological methods. The traditional methods are time consuming, labour-intensive, costly, require mycological expertise and facilities. Immunological methods and diagnostic media have limitation in identifying the aflatoxigenic fungi in that false positives are easy to come by and purifications of samples are a necessary prerequisite. Hence, it is imperative to develop methodologies that are relatively rapid, highly specific and as an alternative to the existing methods. The polymerase chain reaction (PCR) facilitates the in vitro amplification of the target sequence. The main advantages of PCR is that organisms need not be cultured, at least not for long, prior to their detection, target DNA can be detected even in a complex mixture, no radioactive probes are required, it is rapid, sensitive and highly versatile [8]. Many pathogenic organisms have been detected using PCR [9]. The biosynthetic pathway for aflatoxin production by *A. flavus* has been deciphered and genes in the aflatoxin biosynthetic pathway have been identified [11,3]. The gene *afl-2* has been isolated and shown to regulate aflatoxin biosynthesis [12]. Few other genes of the aflatoxin biosynthetic pathway have been cloned and sequenced [13]. Reports on the detection of aflatoxigenic fungi using PCR are rather scanty [4,10,19]. In our studies, the PCR reaction was targeted against aflatoxin synthesis regulatory gene (*aflR1*) since these genes are nearly identical in *A. flavus* and *A. parasiticus* [3], indicating the possibility of detection of both the species with the same PCR system (primers/reaction). However, due to the complexity of the food systems, many food components

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could interfere during PCR interaction while inhibiting the activity of the polymerase [14]. In this study, we describe the use of PCR to distinguish aflatoxigenic fungi present in food samples along with other moulds and microorganisms.

2. Materials and methods

2.1. Microorganisms

The fungal isolates, their source and data on their mycotoxigenic potential are listed in Table 1. The fungal isolates were maintained on potato dextrose agar or on Czapek-Dox Agar media. Cultures were sub-cultured periodically and 5-day-old slant cultures were used in these studies.

2.2. Food commodities

Raw food ingredients including groundnuts and maize were procured from the local market and powdered to pass through a 20 British Standard Mesh (BSM) sieve (700 μm).

2.3. Assessment of fungal load on commodities

Conventional dilution plating techniques were employed to assess the total yeast and mould flora of the raw ingredients [15].

2.4. Primers and PCR chemicals

PCR primers were designed using primer 3 software and were procured from Sigma, Genosys Ltd., UK. Taq polymerase and 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.

2.5. Isolation of fungal DNA

Template DNA was extracted from 0.5 g (wet weight) fungal mycelia harvested from freshly growing cultures in potato dextrose broth (PDB) under stationery conditions. The mycelium was washed twice with phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4) followed by centrifugation. The mycelium was transferred to a mortar, frozen in liquid nitrogen and was ground well. 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 the supernatant transferred to a new centrifuge tube. The supernatant was then extracted twice with phenol:chloroform:isoamylalcohol (25:24:1) and the aqueous layer was precipitated with 2 volumes of isopropanol. The precipitate was resuspended in 200 μl of TE buffer (10 mM Tris–HCl, 1.0 mM EDTA, pH 8.0) [16].

Table 1
List of fungal isolates

Fungal isolate	Source	Mycotoxin
Aspergillus flavus ATCC 46283	Groundnuts seeds	Aflatoxin B1 &B2
Aspergillus ochraceus CFR 221	Coffee curing premises	Ochratoxin A
Fusarium tricinctum NRRL 32998	Standard strain	T-2 toxin, DAS
Fusarium fujikuori NCIM 665	Standard strain	DON, T-2 toxin
Fusarium fujikuroi NCIM 1019	Standard strain	DON, T-2 toxin
Fusarium saubinetti NCIM 851	Standard strain	DON, T-2 toxin
Fusarium spp.	Vegetable (Chow chow)	Zearalenone
Fusarium spp.	Tomato	T-2 toxin
Rhizopus spp.	Traditional starter '*manapu*'	–
Rhizopus chinensis	Traditional starter '*manapu*'	–
Rhizopus arrhizus NCIM 997	Standard strain	–
Aspergillus flavus NCIM 645	Standard strain	Aflatoxin B1 and G1
Aspergillus flavus	Wheat	–
Aspergillus flavus	Maize	–
Aspergillus parasiticus CFR 223	Groundnuts	–
Aspergillus oryzae	Curry spice mix	–
Aspergillus oryzae	Food	–
Aspergillus oryzae	Meat curry spice mix	–
Aspergillus oryzae CFR 225	Soil	–
Aspergillus oryzae	Japanese isolate	–
Aspergillus flavus MTCC 152	Standard strain	Aflatoxin B1 and G1
Aspergillus flavus DCS	Neem (*Asarichta indica*) cake	–
Aspergillus ochraceus CFR 221	Maize	Ochratoxin A
Aspergillus glaucus	Food	–
Aspergillus spp.	Peda-traditional milk sweet	–
Aspergillus flavus	Food	–
Aspergillus niger CFR 224	Soil	–
Penicillium verrucosum MTCC 2007	Peda-traditional milk sweet	–