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Research Article

Detection of *Aspergillus flavus* using PCR method from fungus infested food grains collected from local market

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Abstract: India is an agrarian country two-thirds of its population is engaged directly or indirectly in agricultural activities. In recent years many food borne pathogens have become major threat to public health and safety. The consumption of contaminated food grains or products has been considered to be the leading source of human food borne infections. Surveillance studies have provided data and a better understanding into the existence and spread of food borne pathogens. Aflatoxins produced by *Aspergillus* species are important toxic secondary metabolites known for their impacts on animal and human health, and their effects on the economic loss of key grain and nut crops. Several molecular techniques (including multilocus sequence typing, pulsed field gel electrophoresis, DNA sequencing, multiplex PCR, RAPD, and many more) are available for detection and characterisation of pathogenic microorganisms from food samples, which provide reliable epidemiological data for tracing the source of infections. Present study highlights the possible use of PCR technique, in surveillance and detection of *A. flavus* in fungal infested food grains. The current study was carried out to elucidate the infestation of aflatoxin producing fungus on both kharif (groundnut, rice and maize) and Rabi crops (wheat, gram and soybean). Total 15 samples were collected randomly from local market of Gwalior (M.P). Out of fifteen only nine (60%) samples were found to be *Aspergillus* positive. Seven samples were infested by *Aspergillus flavus* and two by *A. niger*. The selected fungal isolates were identified by amplifying *afl*R gene of *A. flavus* in Thermo Cycler.

Keywords: *Aspergillus flavus,* Aflatoxins, *afl*R, Mycotoxins, Polymerase chain reaction, Primers

Introduction

Aflatoxins are mycotoxins produced by the genus *Aspergillus* that grow on food grains and many other agricultural crops. The molds that are major producer of aflatoxins are *Aspergillus flavus* [1] and *Aspergillus parasiticus* [2]. Aflatoxin B1 is considered as a class 1 human carcinogenic activity [3]. The incidence of aflatoxin in food and feed is relatively high in subtropical and tropical regions where the warm and humid climate provides optimal conditions for the growth of moulds [4]. India is an ideal country for problems of aflatoxin to develop since it has high temperature and high moisture level during the rainy season. There are more than 20 aflatoxins [5] having six major compounds such as aflatoxin B1 (AFB2), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2; [6], aflatoxin M1 (AFM1) and aflatoxin M2 (AFM2). The four AFB1, AFB2, AFG1, and AFG2 are naturally occurring aflatoxins, while aflatoxin M1 (AFM1) and M2 (AFM2) are the hydroxylated metabolites of AFB1 and AFB2 [7, 8]. They are odorless, tasteless and colorless. All aflatoxins are closely similar in structure and form a unique group of highly oxygenated, naturally found heterocyclic compounds.

The aflatoxin B1 biosynthesis involves the twenty three enzymatic reactions starting with acetyl-CoA. A total of fifteen intermediate precursors are recognized in the biosynthesis pathway [9] involving twenty five genes, clustered in a 75-kb DNA region [10]. The sequences of the genes involved in aflatoxin biosynthesis come into view to be highly conserved among *A. flavus* and *A. parasiticus* [11]. There is no specific PCR for any one of the four biologically produced aflatoxins [12]. The late stages of AFB1 synthesis are carried out by two enzymes, a methyltransferase encoded by the *aflP* (*omtA*) gene that converts sterigmatosystin to O-methylsterigmatocystin and an oxidoreductase encoded by the *aflQ* (*ordA*) gene that converts O-methylsterigmatocystin to aflatoxin B1. Bhatnagar *et al.*, in 1991 [13] presented evidence that these later stages of AFB1 and AFB2 synthesis are catalyzed by common enzymes that use separate precursors as substrates for each toxin. Most important requirement for avoiding the consumption of aflatoxin contaminated food is the rapid identification of mycotoxin producing fungi from food samples is usually by traditional dilution plating method. Such method is costly, time consuming and labor intensive and requires expertise. Keeping these problems in mind we

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utilized the already available sequence of PCR primers [14] to identify the *A. flavus* from fungus infected food grains collected from local market.

Materials and Methods

Sample collection

The samples of key grains shown high infestation and crop damage were collected randomly in sterile poly bags from local market of Gwalior (India). From the market located adjacent to the study centre 1kg of each groundnut, gram, maize, rice, soybean and wheat samples were collected by following random sampling technique. From the collected samples, the fungal infected grains were separated out aseptically. Their weight was measured using a digital balance. The percentage of infected nuts and grains present in the collected samples were calculated based on the counts and weight. The same sampling procedure was followed for collecting all the food grains.

Isolation and maintenance of fungus

For fungal isolation direct plate inoculation technique was used using 50 highly fungal infested crop grains obtained from local market located in the vicinity of the research centre. The individual grains were directly inoculated onto potato dextrose agar (PDA) plates and incubated for 5 days at 35°C along with a negative control plate with no inoculation. The observed colonies were subcultured onto Rose-Bengal chloramphenicol media. The colony morphology and appearance of *Aspergillus* strains differ based on the media used. Hence pure cultures of *Aspergillus* species were grown on three fungal differential media - Czapek Extract Yeast Agar (CYA), Malt Extract Agar (MEA), and Czapek Dox Agar (CZA) and incubated at 25°C for seven days [15]. Colony morphological characteristics of isolates were observed both in the top side and bottom side of the media. For identification to species level colony morphology, conidiophores, conidial arrangement (Fig. 2) were observed in microscope after wet mount in lactophenol cotton blue stain. The fungal colonies suspected to be of *A. flavus* were picked and subcultured for DNA isolation. The fungal isolates were maintained on potato dextrose agar media. Cultures were sub-cultured periodically from 5-day-old slant cultures (Fig. 1) isolated from food crop samples. The identification of A. flavus was further confirmed by amplification of *aflR* gene by PCR method.

DNA isolation

The Genomic DNA extraction, from pure fungal cultures was done according to available method [16] with slight modification manually by washing the mycelial pellets twice with 1ml of distilled water. To a 1.5mL Eppendorf tube containing 500μL of lysis buffer (400mM Tris-HCl, [pH 8.0], 60mM EDTA [pH 8.0], 150mM NaCl, 1% sodium dodecyl sulfate), a small lump of mycelia from young culture is added by using a sterile toothpick, with which the chunk of mycelia is disrupted. The tube is then left at room temperature for 15min. After adding 150μL of potassium acetate (pH 4.8; which is made of 60mL of 5M potassium acetate, 11.5mL of glacial acetic acid, and 28.5mL of distilled water), the tube is vortexed and centrifuged at 10000×g for 1min. The supernatant is transferred to another 1.5mL Eppendorf tube and centrifuged again as described above. After transferring the supernatant to a new 1.5mL Eppendorf tube, an equal volume of isopropyl alcohol is added. The tube is mixed by brief vertexing. The tube is centrifuged at 10000×g for 3 min, and the supernatant is discarded. The pellet thus obtained is washed in 300 μL of 70% ethanol. After centrifugation of sample at 10000xg for 1min, the supernatant is discarded. The DNA pellet is air dried and dissolved in 50μL of triple deionized water, and 1μL of this purified DNA is used as a template in 25μL of PCR reaction mixture.

Primers

Primer sequence already available in literature [14] were used to amplify the aflatoxin regulatory gene (*aflR*) fragments of aflatoxigenic fungal genomic DNA. The sequences of the forward and reverse primers of the *aflR* gene used were (5'-AACCGCATCCACAATCTCAT-3') and (5'-AGTGCAGTTCGCTCAGAACA-3'). The primers used in present study cover the region from 540 to 1338 of Aflatoxin regulatory gene with product size of 798 base pairs [17].

Polymerase chain reaction (PCR)

The reaction conditions for the PCR assay were optimized to ensure that all of the target gene sequences were satisfactorily amplified. The polymerase chain reaction was performed in 25 μL of reaction mixture using genomic DNA as template isolated by the method described earlier. The genomic DNA was amplified in Icycler (Bio-Rad, USA) with 30 cycles, each cycle at 94°C for 3 min for denaturation, 0.45 min at 55°C for annealing, 1.25 min at 72°C for extension and a 10 min final extension at 72°C. To check the specificity of primers the PCR was also performed as above using ATCC 367 as a standard of *A. flavus* and the genomic DNA of five different microorganisms (*Staphylococcus aureus, S. epidermidis, E. coli, Clostridium botulinum,* and *Penicillium notatum*).

The amplified PCR products were resolved by gel electrophoresis in a 1.5% agarose (Sigma) gel stained in 0.5 mg/ml ethidium bromide in TBE buffer at 100V for 40min. The DNA bands resolved on agarose gel were visualized in UV transilluminator and photographed. The sizes of the amplicon was estimated after comparing with a commercial 100 bp DNA ladder on agarose gel.

Results

Fungal Infection percentage

Out of the collected 1kg of each crop grains when the infected nuts/grains were separated and weighed they showed an average of 100gm of weight giving an infection weight percentage of 10 and an infection count percentage of 13.

Fungal isolation in basal media

The colour and morphology of colony was used as the first level of identification. From the observations made in infested food grains the fungal growth was seen in all the inoculated plates except negative control. Colonies granular, velvety, or wooly and yellow or yellow-brown; the reverse is golden to red-brown were suspected to be of *A. flavus* while colonies in shades of black and brown colour as *Aspergillus niger*. All the isolates belonging to genus *Aspergillus* were selected for the study. Other characteristic features considered were soluble pigments formation and production of exudates in the media. Based on morphological similarity of fungal colonies from infested crop samples, the number of *A. flavus* and *A. niger*, isolates obtained were 56 and 16 respectively.

Analysis of fungal genes by PCR

In our study the PCR reaction was targeted against aflatoxin synthesis regulatory gene (*afl*R). Genomic DNA isolated from aflatoxigenic *Aspergillus flavus* (ATCC 367) and five other microbes were first used as template and PCR was performed using forward and reverse primer of *afl*R gene. In gel electrophoresis of PCR product and amplicon corresponding to 798 bp in size was seen only in positive control sample of *A. flavus* (ATCC 367) but not in the other samples, which clearly indicated that the primers were specific for *A. flavus* only (Fig. 3). After fidelity test of primers PCR was carried out with fungal isolates, which included 7 strains of *A. flavus*; 2 strains *A. niger* (Table 1). All *A. flavus* strains reacted positively with the *afl*R gene primer set, while no amplification was seen in case of *A. niger* (Fig. 4).

Table 1. Isolated fungal isolates from different food grains.

S.No.	Crop samples	Infestation	Fungal isolates
1.	Groundnut	+	*A. flavus* (I1)
2	Groundnut	-	-
3	Groundnut	-	*A. flavus* (I2)
4.	Gram	-	-
5	Gram	+	*A. flavus* (I3)
6	Gram	-	*A. flavus* (I4)
7.	Maize	+	*A. flavus* (I5)
8	Maize	-	-
9	Maize	-	-
10	Rice	+	*A .flavus* (I6)
11	Rice	-	-
12	Soya bean	+	*A. flavus* (I7)
13	Soya bean	+	*A. niger* (I8)
14	Wheat	-	-
15	Wheat	-	*A. niger* (I9)

Note: + = infected; - = not infected.

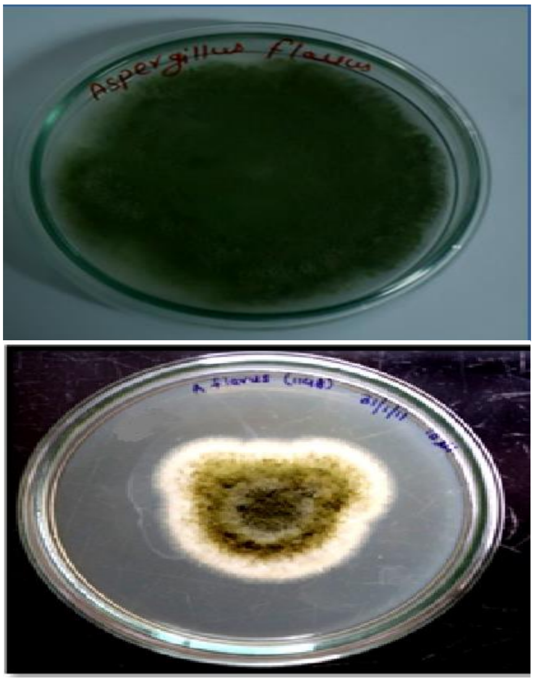

Figure 1. Pure culture of *Aspergillus flavus* isolated from food grains

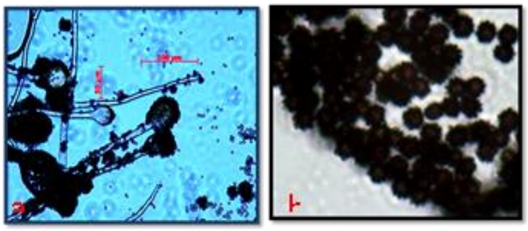

Figure 2. Micro morphological Characteristics of *(a) Aspergillus flavus* (b) *A. niger* under bright field microscope.

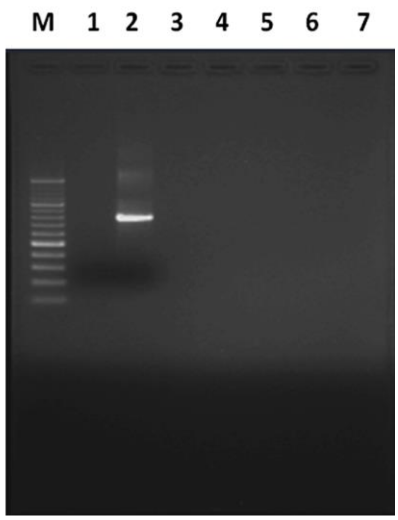

Figure 3: Fidelity analysis of primer by performing PCR using *A. flavus* (ATCC 367) as a standard and other genomic DNA as experimental controls. Lanes: (M) DNA ladder (100bp); (1) Negative control (without template); (2) *A. flavus* ATCC 367; (3) *S. aureus*; (4) *E. coli*; (5) *C. botulinum*; (6) *P. notatum*; (7) *S. epidermidis*

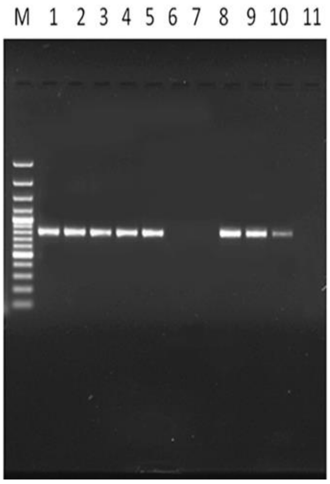

Fig. 4. Agarose gel analysis of PCR products obtained from different fungal species Lanes: (M) DNA ladder (100bp) (1) *A. flavus* ATCC367 (2) *A. flavus* (I1) (3) *A. flavus* (I2) (4) *A. flavus* (I3) (5) *A. flavus* (I4) (6) *A. niger* (I8) (7) *A. niger* (I9) (8) *A. flavus* (I5) (9) *A. flavus* (I9) (10) *A. flavus* (I10) (11) Environmental control (without template)

Discussions

Due to the toxic and carcinogenic properties of aflatoxins, there is an urgent need to develop sensitive, rapid, and specific technique for the identification of aflatoxin producing *A. flavus* from food samples. The DNA amplification technique PCR allows the specific and exponential synthesis of a targeted DNA fragments with the use of small, specifically designed DNA fragments (primers). A number of researchers have used the PCR assay to identify the specific pathogen/organism or contamination of food samples. The PCR is one of the easiest techniques for the detection of any microorganism in samples. Thus we observed that all *7 A. flavus* strains reacted positively with the primer set (Fig. 4) no amplification were observed with DNA from other *Aspergillus* species (*A. niger*), which is quite similar to the observation of Shapira *et al.*, (1996) [18] that identified and sequenced the three genes, versicolorin A dehydrogenase gene (*ver-1*), sterigmatocystin- o-methyltransfersase 1 gene (*omt-1*), and an aflatoxin biosynthesis regulatory gene (*apa-2* now called the *afl*R gene) from *Aspergillus parasiticus*. The aflatoxin gene cluster in *A. flavus* and *A. parasiticus* contains 25 genes reaching about 70 kb. The pathway step, *afl*R, which represents a positive regulatory gene coding for a sequence-specific, is a zinc finger DNA-binding protein. Manonmani *et al.* [19] in 2005 used an indigenously specific primer pair for the aflatoxin regulatory (*afl*R) gene assessed the presence of aflatoxigenic fungi in foodstuffs. In this study DNA isolated from aflatoxigenic *Aspergillus flavus* ATCC 367 was first used as template and PCR carried out with primers for portion of the aflatoxin biosynthetic target gene *afl*R. An amplicon corresponding to 798 bp in size was seen after agarose gel electrophoresis [20]. Here PCR was tested with 9 fungal isolates (Table 1) which included 7 strains of *A. flavus* and 2 strains of *A. niger*.

Conclusions

Several detection methods have been invented and are widely used to detect and differentiate type of pathogens from, outbreak, clinical samples and from epidemiological studies. After discovery of two or more primers method the discriminatory power of the detection or identification technique employed have drastically increased. The primers used in this study amplified only *A. flavus* genes, while the amplification of other *Aspergillus* species (*A. niger*) was not obtained, which clearly indicate that PCR method may be utilised to distinguish *Aspergillus flavus* from other *Aspergillus* species. Furthermore this technique may be applied to the screening of food grains sold in the market for presence of aflatoxin producing fungus *A. flavus*. The negative results of PCR assay may be considered as indicator of food sample free from *A. flavus* infestation. The usefulness of this PCR assay developed so far to screen quality and safety in food industries was already established.

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