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Short Communication

Application of PCR on detection of aflatoxinogenic fungi

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ABSTRACT

Aflatoxins are carcinogenic metabolites produced by several strains of *Aspergillus flavus* group in food and feed. Cluster genes in aflatoxin biosynthesis pathway contain structural, regular and unassigned genes , nor-1 , ver-1 , and omt-1 are structural genes that coding for key enzymes and *aflR* is a regulatory gene that plays a key role in the production of aflatoxin and is affecting on the structural genes and activate transcription. In this study, fourteen strains of *A. flavus* were examined as sample or test group. Three sample of other fungi including *Aspergillus niger*, Penicillium expansum and Fusarium verticillioides as negative controls and one single sample of toxigenic strain of *A. flavus* were studied as positive control , using TLC and PCR with nor-1,ver-1,omt-1 and *aflR* primers. The results showed that three samples of fourteen strains of *A. flavus* were positive using TLC technique and totally twelve samples with the four mentioned primers using in PCR technique showed positive results. None of the other fungal strains using TLC and PCR did show any positive results. The positive control in both techniques was positive. For test sensitivity of the PCR, incubated several spore concentrations of molds accounted in above .Positive results were obtained only with extracts *A. flavus*, even at the lowest spore concentration applied and no DNA amplification observed with other molds even at the highest level. The interpretation of the results revealed that PCR is a rapid and sensitive method.

Keywords: Aflatoxin, *Aspergillus*, PCR, TLC

INTRODUCTION

Aflatoxins are secondary metabolites produced by certain strains of *Aspergillus flavus*, *A. parasiticus*, *A. nomius*, *A. tamarii*, *A. bombycis and A. ochraceoroseus*. These toxins are highly toxic and carcinogenic in animals and humans, leading to hepatotoxicity, teratogenicity, immunotoxicity and even death (Wen *et al* 2004, Motomura *et al* 1999). Among the at least 16 structurally related aflatoxins characterized aflatoxin B1 (AFB1) is the best studied and the most dangerous one (Bhatnagar *et al* 1993, Seherm *et al* 2004), which generally produces by *A. parasiticus* and *A. flavus*. Its economic impact on animal production and passage into human food chain is of great concern globally (Otim *et al* 2005).

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Due to the toxic and carcinogenic properties of AFB1, there is an urgent need to develop rapid, highly specific and sensitive methods for the identification of afla-toxigenic fungi in foods and feeds. Conventional methods used to distinguish among toxingenic and nontoxigenic isolates in the *A. flavus* group involve culturing the fungus in suitable inducing media, extracting aflatoxins with organic solvents, and monitoring their presence by chromatographic techniques (Lin *et al* 1998, Seherm *et al* 2004). The current methods being used for assessing of aflatoxin presence are time-consuming and labor-intensive. Recently, DNA–based detection systems have been introduced as powerful tools for detecting and identifying aflatoxin producing fungi (Geisen 1996). The polymerase chain reaction (PCR) is the method of choice for this purpose (Shapira *et al* 1996). Unique DNA sequences of the respective fungus have to be chosen as primer binding sites concluded that genes involved in the aflatoxin biosynthetic pathway. The AFB1 biosynthetic pathway is generally accepted to be as follows: Acetate, polyketide, norsolorinic acid, averantin, arerufunin, averufin, hydroxyl-versicolorone, versicinal hemiacetal acetate, versicolorinB, versicolorinA, sterigmatocystin-o-methylsterigmatocystin, aflatoxin B1 (Yu *et al* 2004, Motomura *et al* 1999, Wen *et al* 2004). It has been demonstrated that 25 identified genes clustered with in a 70-Kb DNA region in the chromosome are involved in the biosynthesis of AFB1 and their DNA sequences have been published (Yu *et al* 2004, Criseo *et al* 2001, Scherm *et al* 2005). In this study PCR was used for the detection of aflatoxino-genic aspergilli based on the intermediated enzymes including norsolorinic acid reductase encoding gene *nor-1*, the versicolorina dehydrogenase encoding gene *ver-1*, the sterigmatocystin 0-methyl transfrase encoding gene *omt-1* and the regulatory gene *aflR*.

MATERIALS AND METHODS

Fungal strains and culture. Fourteen strains of *A.flarus* and *Aspergillus niger*, *Penicillium expansum* and *Fusarium verticillioide*s were obtained from culture collection of mycology department, school of public health research institute, Tehran University of Medical Science. The strains were cultured on Sabouraud Dextrose Agar (Biolife) and incubated at 26 ºC for 48 to 72 hrs. After the appearance colony, the spores were transferred on the AFAP (*A. flavus* and *A. parasiticus*) medium. All of the plates were incubated at 28-30 ºC for 3-5 days.

Determination of aflatoxin production by chromatography. The entire culture was finely mixed with distilled water and suspension was filtered through Whatman no.1 filter paper. The filtrate was transferred to a separating funnel and extracted with 15-20ml chloroform twice. The obtained extract were centrifuged (1500rpm) for 15min at 4ºC, then the chloroform phase in lower layer was separated and evaporated to dryness. 30µl of the solution was applied to thin layer chromatography (TLC) plate. An aflatoxigenic strain was used as positive control.

DNA extraction. The strains were transferred to BHI broth and incubated under conditions of continuous shaking (150 rev/min) for 72 hrs. 1ml of each sample was transferred to Eppendorf tube and centrifuged for 15 min at 5000 rpm. The supernatant fluid was discharged and add lysis buffer (Tris-HCL 50 mM pH=8, SDS 1%, Nacl 100mM, EDTA 50 mM, proteinase K 20 µl to 200µl) to the tube equal volume of the composition sediment in it and incubate for at least 4 hrs at 56 ºC. Equal volume of the material in the tube added phenol and mixed well by inversion. Centrifuge at 13000 rpm for 15 min. Remove all aqueous layer (top layer) and transfer in a new tube. Add phenol: chloroform (1:1) in tube equal volume of the tube containing. Centrifuged at 13000 rpm for 15 min and remove all

aqueous layer and transfer in a new tube. Add chloroform in the tube, equal volume of the tube containing mix them well by inversion and centrifuge at 13000 rpm for 5 min. Remove all aqueous layer and transfer in a new tube and sodium acetate was added 1:10 volume of the tube containing and mix well. Add to them ethanol two fold of material in tube. This solution was placed on ice for 20 min and centrifuged for 15 min at 13000 rpm. Discharge containing of tube and 200 µl of 70% ETOH, centrifuged for 5 min at 13000 rpm. Pour off ETOH and drying tubes then add 50 µl distilled water to them.

PCR. Four published primer sets were used for the specific detection of *nor-1*, *ver-1*, *omt-1* and *aflR* genes (Scherm *et al* 2005) as follow: Nor-1F 5′-ACC GCT ACG CCG GCA CTC TCG GCAC-3′ and Nor-1R 5′-GTT GGC CGC CAG CTT CGA CAC TCC G-3′, Ver-1F 5′-ATG TCG GAT AAT CAC CGT TTA GAT GGC-3′ and Ver-1R 5′-CGA AAA GCG CCA CCA TCC ACC CCA ATG-3′, Omt-1F 5′-GGC CCG GTT CCT TGG CTC CTA AGC-3′ and Omt-1R 5′-CGC CCC AGT GAG ACC CTT CCT CG-3′, and *aflR*F 5′-TAT CTC CCC CCG GGC ATC TCC CGG-3′ and *aflR*R 5′-CCG TCA GAC AGC CAC TGG ACA CGG-3′. The 400, 895, 1232 and 1032 bp fragments were amplified respectively. PCR was performed in 25µl containing 2.5µl of 10XPCR buffer, 0.75µl of 25mM $MgCl_2$, 0.5µl of 10mM dNTPs, 0.625µl each primer, 5U *Taq* DNA polymerase, 2µl of extracted DNA as template and 17.5µl of sterile distilled water. A total of 35 cycles was started by heating at 94 ºC for 10min and continued by denaturation 1min at 94 º C, annealing 2min at 65 º C, elongation 2min at 72 ºC and a final extension 5min at 72 ºC. Visualization of amplified products was done by UV illumination after electrophoresis on 1% agarose gel and ethidium bromide staining.

RESULTS AND DISCUSSION

A. flavus strains growth on the AFAP (*A. flavus* and *A parasiticus*) medium after incubation at 28 ºC to 30 ºC for 3-5 days and other molds growth on sabouraud dextrose agar (Biolife) after 48 to 72h incubation at 26ºC. DNA of all fungi were extracted. Extraction of DNA was done by phenol-choloroform method that is very trust in spite of time consuming and its hardship stages.

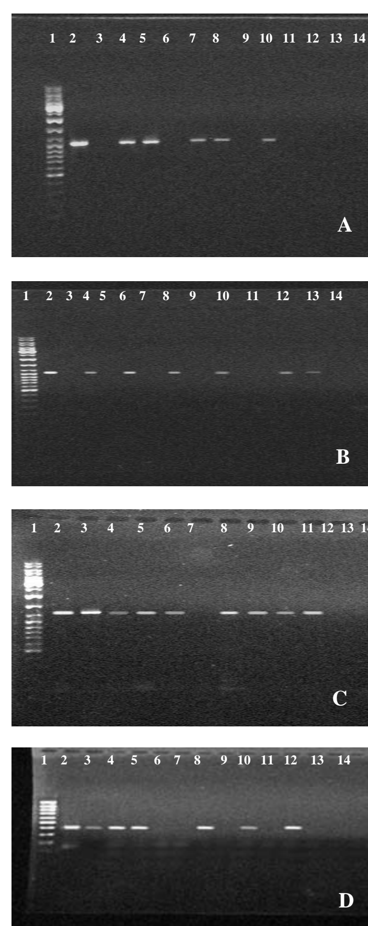

Figure 1. Gel electro-phoresis analysis PCR products using primers and DNA extracted from 14 strains of *A. flavus*. (**A**): *Lanes* 1-14 DNA of *A. flavus* strains using *aflR* primer with 1032bp. (**B**): *Lanes* 1-14 DNA of *A. flavus* strains using *ver-1* primer with 895bp. (**C**): *Lanes* 1-14 DNA of *A. flavus* strains using *omt-1* primer with 1235bp. (**D**): *Lanes* 1-14 DNA of *A. flavus* strains using *nor-1* primer with 400bp.

PCR was applied using four sets of primer for different genes involved in aflatoxin biosynthetic pathway. Figure 1 (a-d) show the PCR products obtained from each primer. Bands of the fragments of *aflR*, *omt-1*, *ver-1* and *nor-1* genes can be visualized at 1032, 1232, 895 and 400-bp, respectively. Four strains, 1, 3, 7 and 9 shows a

similar pattern indicating the presence of the four genes and other strains presented varying patterns. The results obtained by TLC indicated among examined. Strains only three (1, 3 and 7) were aflatoxin-produce fungi. The results obtained by PCR and TLC are compared in table 1.

Table 1. This table indicates a comparison between the conventional and molecular methods (TLC and PCR) on aflatoxin production.

samples	**PCR results**				**Aflatoxin production by TLC* method**
Strain No.	***afl R***	***omt-A***	***ver-1***	***nor-1***	**Alfaotoxin production**
1	+	+	+	+	Positive
2	-	+	-	+	Negative
3	+	+	+	+	Positive
4	+	+	-	+	Negative
5	-	+	+	-	Negative
6	+	-	-	-	Negative
7	+	+	+	+	Positive
8	-	+	-	-	Negative
9	+	+	+	+	Negative
10	-	+	-	-	Negative
11	-	-	+	+	Negative
12	-	-	+	-	Negative
13	-	-	-	-	Negative
14	-	-	-	-	Negative
Control +	+	+	+	+	Positive
A. niger	_	_	_	_	Negative
P.expansium	_	_	_	_	Negative
F. verti-cillioides	_	_	_	_	Negative

* *TLC:* Thin layer chromatography.

No DNA amplification observed with Aspergillus niger, Penicillium expansium and Fusarium verticillioides even at the highest level that is indicated high specificity of PCR. To determination the PCRs sensitivity, lower concentration of spores were tested. DNA amplified only in *A. flavus* even at the lowest spore level.

The important aim of this study is to standardize and optimizes a PCR method for detecting and probing the fungi, which produce aflatoxin by effective genes in biosynthesis pathway. To approach this goal, we have surveyed on fourteen strains of *A. flavus* as a test sample and three samples of other fungi as well, such as A.ngier, P.expansium, F.verticillioides, which were assumed a positive control through TLC technic and PCR method, working with *nor-1*, *ver-1*, *omt-1*, *aflR* primers. *Nor-1*, *ver-1*, *omt-1* are three structural genes in cluster genes in biosynthesis aflatoxin pathway that coding for key enzymes in production of aflatoxin, thus they are essential for aflatoxin production (Yu *et al* 2004). Phenol-chloroform method was selected for DNA extraction, this method is considered as the most complete and reliable method which eliminates interfering objects such as particles, proteins and lipids in DNA extraction. After extraction and DNA measurement by using spectrophotometery method and by nano drops instrument, the temperature program of each PCR cycles, the specified time and amount of each subjects were optimized by using previous studies (Chen *et al* 2002, Griseo *et al* 2001, Geisen 1996, shapira *et al* 1996, Farbert *et al* 1997.

The result revealed that applied conditions were set up perfectly, as though each primer formed sharp and distinct bands in its specific area. Other studies (Ehrlich *et al* 2003, Cary *et al* 2002, Chang *et al* 2000, Takahshi *et al* 2002, Floherty & Payne 1997, Chony *et al* 1999a, b) suggest that regulation of aflatoxin biosynthesis in *Aspergillus* spp. involves a complex pattern of positive and negative acting transcriptional regulatory factors, which are affected by environmental and nutritional parameters. The results showed that three samples of fourteen strains of *A. flavus* were positive using TLC technique and totally twelve samples with the four mentioned primers using in PCR technique showed positive results. None of the other fungal strains using TLC and PCR did show any positive results. The positive control in both techniques was positive. For test sensitivity of the PCR, incubated several spore concentration of molds accounted in above. Positive results were

obtained only with extracts A. flavus, even at the lowest spore concentration applied and no DNA amplification observed with other molds even at the highest level. The same result has been achieved by using PCR and multiplex PCR procedures (Shapira *et al* 1996, Criseo *et al* 2001). The interpretation of the results revealed that PCR is a rapid and sensitive method (sensitivity 100%, specificity 75%) in diagnosis of aflatoxinogenic molds but, this technique (PCR) can not differention between toxigenic and nontoxigenic fungi. Geisen (1996) suggests that the lack of aflatoxin production could also be due to simple mutations including substitution of some bases and Liu & Chu 1998 suggest that a variety of different physiological conditions affecting aflatoxin biosynthesis. In this study observed we can approach to the PCR method as a screening test for primary isolation due to its speed and high sensitivity (100%). The positive samples should be investigated for further examination such as chromatography and RT-PCR. RT-PCR method is a complementary assay to PCR and survey on gene presentation. Sweeney *et al* 2000, Mayer *et al*, 2003 suggested that the presence or lack of mRNA could permit direct differentiation between them. In this regard multiplex RT-PCR with the advantage of having a unique response to the expression of several genes enclosed in the aflatoxin biosynthesis pathway and an experimental real-time RT-PCR could be correlated to the growth kinetics of the fungus and to the presence of AFB1 were designed, however, none of these methods has yet been applicable to differentiate between toxinogenic and non-toxinogenic strains of *A. flavus* group.

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