

Application of PCR in the Detection of Aflatoxinogenic and Non-aflatoxinogenic Strains of *Aspergillus Flavus* Group of Cattle Feed Isolated in Iran

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Abstract

Aflatoxins are among the most important Mycotoxins that are mainly produced by various *Aspergillus* species, specially *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxins are carcinogenetic and immunosuppressive, so that can lead to acute liver damage, cirrhosis of the liver and hepatocarcinoma induction. Consuming the feed contaminated by *Aspergillus* puts humans and animals under the danger of Aflatoxins that are considered as an important threats for human and animal health. The purpose of the present study was to make distinction between Aflatoxinogenetic and non-Aflatoxinogenetic strains and *Aspergillus Flavus* using PCR and TLC and the expression of five Aflatoxin biosynthesis genes including *aflD (nor-1)*, *aflP (omtA)*, *aflO (omtB)*, *aflQ(ordA)*, *aflR* in 40 strains was investigated using PCR. In this study, a number of 40 *Aspergillus flavus* strains from 67 species of cattle feed from 21 industrial warehouses of various areas of Tehran and Alborz were used. After isolation and culture in exclusive environment of yeast extract of sucrose agar, the isolated *Aspergillus* strains were investigated by microscopic and macroscopic methods. In order to make distinction between Aflatoxinogenetic and non-Aflatoxinogenetic strains, PCR method and TLC techniques were used. The results showed that only 7 strains (1, 3, 5, 14, 22, 34, and 38) were Aflatoxin-producers fungi and the rest 33 samples were non-Afatoxin-producers fungi. Since *Aspergillus flavus* is the main contaminator of cattle feed, there is a need to develop a simple, rapid and sensitive method to identify Aflatoxigenetic fungi, particularly between Aflatoxinogenetic and non-Aflatoxinogenetic strains of AF.

Keywords: Aflatoxinohenetic, non-Aflatoxinogenetic, *Aspergillus flavus* strains, PCR, TLC, Cattel feed

1. Introduction

Fungi growth in stored feed is one of the threatening factors of human health (Richard et al., 2003). The use of unhealthy cattle feed causes distortion in health cycle of cattle, people and diaries (Humans, Organization, & Cancer, 2002). Various studies on cattle feed have shown that the contamination of cattle feed by fungi, particularly *Aspergillus* species, leads to the creation of Aflatoxin (J. W. Bennett & Klich, 2003; Kamei & Watanabe, 2005).

Aflatoxins are considered as secondary fungal metabolites that are easily created along the growth and storage of feed and are mainly produced by *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius* (K. C. Ehrlich, Montalbano, & Cotty, 2003; Varga, Frisvad, & Samson, 2009; Yu et al., 2004; Yu, Woloshuk, Bhatnagar, & Cleveland, 2000).

Goldbatt reported that the death of more than one thousands turkeys in England was due to the contamination of Brazilian peanut meal poultry feed to *Aspergillus* and prtoducing Aflatoxin. This disease is caused by toxin produced by *Aspergillus flavus* on birds' feed (Wogan & Pong, 1970). This toxin in 1961 led to more awareness regarding damages resulting from fungi toxins as the contaminator of food and causing disease or even death in

humans and animals and years between 1960 and 1975 were the golden years of studies on fungi toxins, since scientists conducted many studies on these toxigenic factors (J. W. Bennett & Klich, 2003).

Aflatoxins, compared to other fungi toxins, due to carcinogenicity and acute toxicity effects, are more important. Among the detrimental effects of Aflatoxins, we can mention weakening and destroying the immune system, genetic mutations and cancer (Chu, 1991; Richard et al., 2003). There are four main types of Aflatoxins including: AFB1, AFG1, AFB2 and AFG2 (J. Bennett, Kale, & Yu, 2007; Murphy, Hendrich, Landgren, & Bryant, 2006). Here, AFB1 is the most toxic one (Van Egmond, Schothorst, & Jonker, 2007; Yu, Bhatnagar, & Ehrlich, 2002). Among effective factors in producing Aflatoxins, we can refer to genetic and environmental factors such as the type of fungi, type of medium, humidity, temperature, growth, storage, ventilation, light, carbon source, and pH. At least 23 enzymatic reactions contribute in Aflatoxin synthesis path. The synthesis of this toxin is done through a series of oxidation and reduction reactions. The Aflatoxin biosynthetic pathway involves approximately 25 genes clustered in a 70 kb DNA region (Bhatnagar, Cary, Ehrlich, Yu, & Cleveland, 2006; Scherm, Palomba, Serra, Marcello, & Migheli, 2005).

Most of these genes are regulated by the specific path of the protein binding to DNA (AflR) and are produced by *aflR* gene (Chang, Horn, & Dorner, 2005; Yu et al., 1995). Due to the toxic and carcinogenic characteristics of Aflatoxins, there is an immediate need for a rapid method. There are sensitive and particular methods for detecting Aflatoxigenic matters in feed and the conventional methods to make distinction between toxigenic and non-toxigenic isolations in *Aspergillus flavus* including fungi culture in suitable medium, extracting Aflatoxins by organic solvents and monitoring their presence by chromatographic techniques (Abbas et al., 2004; Fente, Ordaz, Vazquez, Franco, & Cepeda, 2001; Sforza, Dall'Asta, & Marchelli, 2006). The current methods used for monitoring the presence of Aflatoxins are time consuming and labor intensive.

Recently, DNA-based detection systems are introduced as powerful tools to detect and identify Aflatoxin (Geisen, 1996). The Polymerase Chain Reaction (PCR) is among the selected methods for this purpose (Criseo, Bagnara, & Bisignano, 2001; Färber, Geisen, & Holzappel, 1997; Shapira et al., 1996; Sweeney, Pàmies, & Dobson, 2000). Unique DNA sequences of the respective fungus have to be chosen as primer binding sites concluded that genes involved in the Aflatoxin biosynthetic pathway (Geisen, 1996; Scherm et al., 2005). The purpose of the present study was to make distinction between Aflatoxinogenetic and non-Aflatoxinogenetic strains using PCR and TLC and the expression of *aflD* (*nor-1*), *aflP* (*omtA*), *aflO* (*omtB*), *aflQ* (*ordA*), *aflR* in 40 strains was investigated using PCR.

2. Materials and Methods

2.1 Fungal Strains and Culture

A number of 40 *Aspergillus flavus* strains from a collection of 67 species from cattle feed including maize (A), domestic barley (B1), imported barley (B2), wheat bran (D) and soybean meal (E) were collected from 21 industrial warehouses of Tehran and Alborz.

Strains were cultured on Sabouraud dextrose agar (SDA) medium containing chloramphenicol (to prevent the growth of bacteria and yeasts) and plates were incubated at the temperature of 28-30 °C for 3-5 days. After the emergence of colony, the fungal spores were transmitted to yeast extract of sucrose agar (YES) (Samson, Hoekstra, & Frisvad, 2004). All plates were incubated at the temperature of 25 °C for one week. Then, the growing strains in each plate were put under microscopic and macroscopic investigations using valid identification keys (Klich & Pitt, 1988).

2.2 Determination of Aflatoxin Production by Chromatography

Therefore, 500 µl of samples were mixed by 250 µl of chloroform. Then, they were dotted on TLC plate of silica gel, so that the distance of dots with the lower margin of chromatography plate was approximately 2 cm and these dots were dried at the temperature of 27 °C. After that, 50 ml extraction solvent (containing 40 ml toluene, 7/5 ml methanol and 2/5 ml acetic acid) were added to the solvent tank, so that the depth of solvent should be at the bottom of the tank, less than 2 cm. The TLC plate is placed in tank and let the solvent be 4-5 cm higher than the dotted line (about 15 to 20 minutes). Then, bring the TLC plate out of the solvent tank to be dried and observable under ultraviolet light (365 nm). An Aflatoxigenic strain was used as a positive control.

2.3 Molecular Characterization

2.3.1 Detection of Aflatoxin Genes *aflD* (*nor-1*), *aflP* (*omtA*), *aflO* (*omtB*), *aflQ* (*ordA*) and *aflR*

In this study, common naming system of Aflatoxin genes was used by (Cary, Ehrlich, Bland, & Montalbano, 2006; K. Ehrlich, Yu, & Cotty, 2005; Yu et al., 2004). The performance of these five genes is indicated in Table 1.

Table 1. Aflatoxin biosynthetic genes and functions^a

Old name	New name	Enzyme/product	Function in the pathway
<i>nor1</i>	<i>aflD</i>	NOR reductase	<i>norsolorinic acid (NOR)</i> → <i>averantin (AVN)</i>
<i>omtA</i>	<i>aflP</i>	O-methyltransferase A	<i>sterigmatocystin (ST)</i> → <i>O-methylsterigmatocystin (OMST)</i>
<i>omtB</i>	<i>aflO</i>	O-methyltransferase B	<i>DHDMST (dihydrodemethylsterigmatocystin)</i> → <i>DHST (dihydrosterigmatocystin)</i>
<i>ordA</i>	<i>aflQ</i>	Oxydoreductase	<i>O-methylsterigmatocystin (OMST)</i> → <i>AFB1</i> and <i>AFG1</i> , <i>dihydro-Omethylsterigmatocystin (DHOMST)</i> → <i>AFB2</i> and <i>AFG2</i>
<i>aflR</i>	<i>aflR</i>	Transcription activator <i>AflR</i>	Pathway regulator

^aClustered pathway genes in aflatoxin biosynthesis (Cary et al., 2006; K. Ehrlich et al., 2005; Yu et al., 2004).

2.4 DNA Extraction

To extract DNA, some microliters of spore suspension of *Aspergillus* isolate were kept, transferred to the plate containing YES, and a one-week colony is employed. 500 microliters lysis buffer (containing 1 molar Tris-HCl (PH=8), 0.5 molar EDTA (PH=8) and 7.45 g KCl), a pile of about 60 mg mycelium was added from *Aspergillus* colony and then crushed by hand and vortex for 45 seconds and finally centrifuged for 10 minutes at 5000 g. The supernatant liquid was transferred to new fresh tube, and 300 microliters cold isopropanol (kept below -20°C) was added and finally cell lysis and isopropanol were mixed through multiple reversal activities of microtube and centrifuged for 10 minutes at 12000 g. The supernatant liquid was discarded and about 0.8 microliter 70 degree alcohol was added to sediment and after 15 minutes was incubated at 37°C. Eventually, 50 microliters deionized distilled water was added to the remaining sediment, and then DNA was mixed with distilled water by gently tapping. The resulting liquid is frozen and stored at -20°C as a pure DNA solution.

2.5 PCR Amplification

In the present study, five pairs of primers were designed based on *Aspergillus flavus* sequences from *aflD (nor-1)*, *aflP (omtA)*, *aflO (omtB)*, *aflQ (ordA)*, *aflR* using OLIGO7 software (Scherm et al., 2005). Oligonucleotides were made by Macro-Gen Company. The primer sequences are presented in Table 2.

Table 2. Sequences of the nucleotide primers used in this study

Primer code	Target gene	Primer sequences	PCR product size (bp)	Accession no
<i>AflD-1for</i>	<i>aflD(nor-1)</i>	5'- CTCATCACACGCAGGCATCGG -3'	702	FN398169.1
<i>AflD-2rev</i>		5'- AGATGCCTGCCACACTGTCT -3'		
<i>AflP-1for</i>	<i>aflP(omtA)</i>	5'- CCCATCTCGATAGCGCCTG -3'	611	FN398191.1
<i>AflP-2rev</i>		5'- GCCACCCATACTAGATCAAAGC -3'		
<i>AflO-1for</i>	<i>aflO(omtB)</i>	5'- TTACGATTTGATGGAGCAGG -3'	358	HM355030.1
<i>AflO-2rev</i>		5'- AGGTTCTCTTGCTACAG -3'		
<i>AflQ-1for</i>	<i>aflQ(ordA)</i>	5'- AACATTCTCTGCCTCATCACT -3'	445	Ay510451.1
<i>AflQ-2rev</i>		5'- TCGCTCTGGCTTGAACACC -3'		
<i>AflR-1for</i>	<i>aflR</i>	5'- AGAGCTACTGAACGTCCCAT -3'	1458	AF441430.2
<i>AflR-2rev</i>		5'- ATCAGGTTGCACGAAGTGTCC -3'		

About 5 microliters of extracted DNA, 1 microliters of forward and reverse primers, 10 microliters of PCR master mix prepared by Amplicon Company (containing 0.2 U/μl of Taq DNA polymerase, 0.4 millimolar of dATP and dNTP (dTTP, dCTP, dGTP and 3 millimolar of MgCl₂) and necessary amount of sterile deionized distilled water (ddH₂O) were added until achieving the final volume of 20 microliters. The heating program of PCR was done according to 错误!未找到引用源。 .

Table 3. Heat program used for PCR

1 cycle		34 cycle				1 cycle				
PCR steps	Initial denaturation	Denaturation		Annealing		Extention		Final extention		
	Tm	Time	Tm	Time	Tm	Time	Tm	Time	Tm	Time

<i>aflD</i>	95 °C	3 min	95 °C	30 sec	61.4°C	40 sec	72°C	30 sec	72°C	7 min
<i>aflP</i>	95 °C	3 min	95 °C	30 sec	60°C	45 sec	72°C	45 sec	72°C	7 min
<i>aflO</i>	95 °C	3 min	95 °C	30 sec	52.4°C	30 sec	72°C	30 sec	72°C	7 min
<i>aflQ</i>	95 °C	3 min	95 °C	30 sec	57°C	30 sec	72 °C	45 sec	72°C	7 min
<i>aflR</i>	95 °C	2 min	95 °C	30 sec	58 °C	45 sec	72°C	1:30 min	72°C	7 min

3. Results and Discussion

Aspergillus flavus strains were grown on yeast extraction of sucrose agar at the temperature of 25 °C for one week. In the present study, another set of primers exclusive for *aflR* was used. This gene has a very important role in biosynthesis path of Aflatoxin by regulating the activities of other structural genes such as *nor-1*, *omtA*, *omtB* and *ordA* (Chang, Yu, Bhatnagar, & Cleveland, 1999; Woloshuk et al., 1994).

PCR was applied for these genes using five sets of primers. Figure 1 (A-E) shows products of PCR obtained from each primer. A series of bands from *aflD* (*nor-1*), *aflP* (*omtA*), *aflO* (*omtB*), *aflQ* (*ordA*) and *aflR* can be observed in 702, 611, 358, 445, and 1458 bp. Nine strains (1, 2, 3, 5, 13, 14, 22, 34 and 38) show a similar pattern that indicates the presence of all five genes and other strains show different patterns. The obtained results by TLC were investigated. Only 7 strains (1, 3, 5, 14, 22, 34 and 38) were Aflatoxin-producers fungi and other 33 samples were non-Aflatoxin producers fungi. TLC method has shown a clear distinction between Aflatoxin-producing and non-producing *Aspergillus flavus*. The results obtained by PCR and TLC are compared in 错误!未找到引用源. .

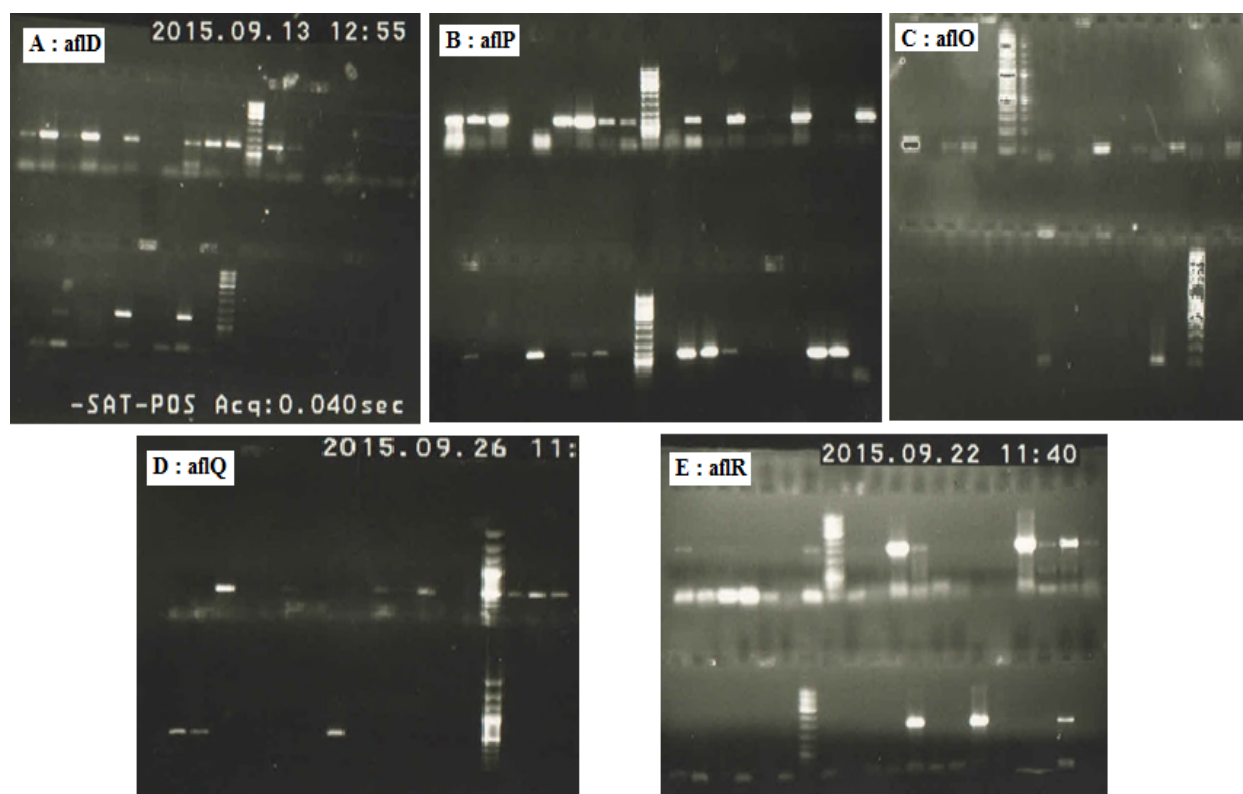


Figure 1. The images of gel electrophoresis of PCR products for expression of genes (*aflD*, *aflP*, *aflO*, *aflQ* and *aflR*) of *Aspergillus* isolated from cattle feed in 1% agarose gel and ladder 1kb

Table 3. This table indicates a comparison between the conventional and molecular methods (TLC and PCR) on aflatoxin production

Sample	PCR results	Aflatoxin production by TLC* method
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Sample Type	Strain No.	<i>aflR</i>	<i>aflP</i>	<i>aflO</i>	<i>aflQ</i>	<i>aflD</i>	Alfaotoxin production
1:B2	1	+	+	+	+	+	Positive
1:B2	2	+	+	+	+	+	Negative
1:B2	3	+	+	+	+	+	Positive
1:E	4	-	-	+	+	+	Negative
2:B1	5	+	+	+	+	+	Positive
2:B2	6	-	+	-	+	-	Negative
2:E	7	+	+	+	+	-	Negative
3:B2	8	+	-	+	+	-	Negative
3:E	9	+	+	+	+	-	Negative
4:A	10	-	+	+	+	-	Negative
4:A	11	-	-	+	+	-	Negative
5:B2	12	+	-	+	+	-	Negative
5:D	13	+	+	+	+	+	Negative
5:E	14	+	+	+	+	+	Positive
6:E	15	+	+	+	-	+	Negative
7:A	16	-	+	+	+	+	Negative
7:A	17	-	-	+	+	-	Negative
7:D	18	+	+	+	-	-	Negative
8:B1	19	+	+	+	-	-	Negative
9:A	20	+	+	+	-	-	Negative
9:B2	21	+	+	+	+	-	Negative
9:D	22	+	+	+	+	+	Positive
9:D	23	+	+	-	-	-	Negative
9:D	24	-	+	+	-	+	Negative
10:B1	25	+	-	+	+	+	Negative
10:E	26	+	+	+	-	-	Negative
11:A	27	-	+	+	+	-	Negative
11:E	28	+	-	+	+	-	Negative
12:B1	29	+	+	+	+	-	Negative
13:B1	30	+	+	+	-	-	Negative
13:D	31	+	+	+	+	-	Negative
14:A	32	-	+	+	+	-	Negative
15:A	33	+	-	+	+	-	Negative
15:B2	34	+	+	+	+	+	Positive
15:D	35	-	+	+	+	+	Negative
19:B2	36	-	-	+	+	-	Negative
19:D	37	-	+	+	+	-	Negative
20:A	38	+	+	+	+	+	Positive
20:A	39	-	+	-	-	-	Negative
20:D	40	-	-	+	+	-	Negative
Control		+	+	+	+	+	Positive

*TLC: Thin layer chromatography.

The purpose of the present study was to standardize and optimize PCR method to make distinction between Aflatoxinogenetic and non-aflatoxinogenetic strains of *Aspergillus flavus* that produce Aflatoxins by effective genes in biosynthesis path. For this purpose, we worked on 40 strains of *Aspergillus flavus* selected from 67 species of cattle feed from 21 warehouses of Tehran and Alborz as the samples of this study and an standard strain of *Aspergillus flavus* 5004 as a positive control to examine TLC and PCR. The procedure was conducted by *aflD* (*nor-1*), *aflP* (*omtA*), *aflO* (*omtB*), *aflQ* (*ordA*) and *aflR*. *Nor-1*, *omtA*, *omtB* and *ordA* are four structural genes in gene cluster in Aflatoxin biosynthesis path that code key regulating enzymes in Aflatoxin production. As a result, they are necessary for Aflatoxin production (Yabe & Nakajima, 2004; Yu et al., 2004). After DNA extraction, the heating program of each PCR cycle was optimized within specific time using previous studies (Criseo et al., 2001; Färber et al., 1997; Geisen, 1996; Shapira et al., 1996).

The results showed that 5 primers, constituted sharp and distinct bands in certain area. Another studies show regulating Aflatoxin biosynthesis in *Aspergillus* containing a complex pattern of positive and negative transcription regulating factors that are under the influence of nutritional and environmental parameters (Chang, Yu, Bhatnagar, & Cleveland, 2000; K. C. Ehrlich et al., 2003; Flaherty & Payne, 1997; Takahashi et al., 2002). The results showed that seven samples of 40 *Aspergillus flavus* species were positive using TLC and in general, 40 samples with the five mentioned primers were positive using PCR technique and positive control in both methods was positive, as well. The same results were obtained using PCR and multiplex PCR (Criseo et al., 2001; Shapira et al., 1996). The results showed that PCR is a sensitive, rapid and specific method in Aflatoxinogenetic molds detection, but it cannot make distinction between toxigenic and non-toxigenic fungi. According to the results of this study regarding non-Aflatoxin producing strains, there is not any relationship between the obtained results by PCR and conventional methods. Lack of Aflatoxin production is related to an incomplete pattern in PCR. This proposes that various types of mutations can deactivate Aflatoxin biosynthesis path of these strains. Geisen (1996) stated that the lack of Aflatoxin production can be the result of substitution of some bases (Geisen, 1996). Also, Liu and Chu (1998) showed that various physiologic conditions can be effective in Aflatoxin biosynthesis (Liu & Chu, 1998). In this study, it was observed that we can refer to PCR as a screening test for initial isolation regarding high sensitivity and speed (100%). The positive samples should be more investigated such as chromatography and RT-PCR. RT-PCR is a complementary measurement of PCR and the presence of gene. Mayer et al. (2003) and Sweeney et al. (2000) suggested that the presence and absence of mRNA could allow direct distinction between them (Mayer, Färber, & Geisen, 2003; Sweeney et al., 2000).

In this regard, multiplex RT-PCR by having the advantage of unique response to expressing several genes enclosed in the biosynthesis of Aflaxotin and real experimental time of RT-PCR can be used for fungi growth kinetic and the presence of designed AFB simultaneously. However, none of these methods has yet been applicable to make distinction between non-taxogenic and taxogenic strains of *Aspergillus flavus*.

4. Conclusion

Since investigations show that the highest pollution of cattle feed is related to toxigenic *Aspergillus*, therefore, there is need for developing a simple, rapid and sensitive method to detect Aflatoxigenic fungi, particularly, making distinction between Aflatoxin-producing and non-producing strains of AF. As a result, we can prevent the entrance of Aflatoxin to the health cycle of human and cattle.

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