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An investigation into the effect of aflatoxin genes *aflD, aflO* and *aflJ* on sterigmatocystin in cattle feed using PCR technique

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ABSTRACT

Sterigmatocystin is amongst the ultimate precursor of aflatoxin in aflatoxin-producing fungi. These carcinogenic and mutagenic compounds may cause teratogenicity and immunosuppression. Human and animals are susceptible to lead aflatoxin poisoning when their food source is contaminated by Aspergillus. This study aims to investigate the effect of aflatoxin genes aflD, aflO and aflJon sterigmatocystin, the ultimate precursor of aflatoxin, in cattle feed. To conduct the study, 121 samples of cattle feed were collected from 21 industrial animal husbandary in Tehran and Alborz provinces, and then were isolated and cultured based on macroscopic and microscopic methods. Moreover, PCR technique was also used to undertake a molecular investigation into the isolated Aspergillus. To identify the relationship between aflatoxin genes with sterigmatocystin, 20 samples of cattle feed, containing one, two or three positive aflatoxin genes were randomly selected, examined under the ultraviolet light and finally, evaluated using thin-layered chromatography. The results indicate that 55.37% of 121 samples of the cattle feeds contaminated by Aspergillus fungi. Among these isolated samples, 37.31% has aflD, 90.01% has aflO and 34.32% has aflJ and the findings of TLC also suggest that only 2 out of 20 cattle feeds show high sterigmatocystin content while others are less infected. These two samples were positive for all three aflatoxin genes, and a direct association was also found between aflatoxin genes aflO, aflJ and aflD and sterigmatocystin. Controlling cattle feeds contaminated by Aspergillus Fungi can secure them against sterigmatocystin and aflatoxin contamination and prevent them from entering the human and animal health cycle.

Keywords: Aflatoxin, Sterigmatocystin, Aspergillus, PCR, TLC, Cattle feed

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INTRODUCTION

Mycotoxins are produced in grains like corn, sorghum, barley, wheat, cottonseed meal, peanut and fodder before and at-harvest at extreme humidity [1].The term mycotoxin was coined in 1962 in the aftermath of an unusual veterinary crisis near London, England, during which approximately 100,000 turkey poults died. Once this disease was linked to the toxin produced by *Aspergillus Flavus* on poultry feeds, it sensitized scientists to the possibility that other mold metabolites might be deadly [2]. The years between 1960 and 1975 have been termed the mycotoxin gold rush since scientists conducted a lot of research on these toxigenic agents [3]. Mycotoxins are secondary metabolites produced by filamentous fungi and have four basic types of toxicity including acute, chronic, mutagenic and teratogenic [1,

4].The most commonly described effect of acute mycotoxin poisoning is deterioration of liver or kidney function, which in extreme cases may lead to death. Chronic mycotoxin, however, may cause cancer or immunodeficiency [5]. Various types of mycotoxins produced by *Aspergillus* include aflatoxins, ochratoxin, citrinin, patulin, penicillin acid and sterigmatocystin [3, 6], among which sterigmatocystinsis the most important of all, produced by *Aspergillus nidulans* species and *Aspergillus versicolor* species[7]. Although sterigmatocystinis a potent hepatocarcinogen similar to aflatoxin B1 in terms of their chemical properties, and is produced by over 20 types of *Aspergillus* not as a mediator, but as a final product, this compound is amongst the final aflatoxin precursor in toxigenic fungi [7, 8]. The genes involved in the biosynthesis of STC in *Aspergillus nidulans* constitute a 60kbp gene cluster [9]. Low acute toxicity was observed in sterigmatocystin, yet the major concern is for its carcinogenic risk, which is 1.10 higherthan that of aflatoxin B1[10]. This toxin is isolated in from green coffee beans, wheat, corn, cattle feed, cheese and nuts [11]. None of the developing countries is reported to haveadopted a law to determine the level of sterigmatocystin, emphasizing the need for rules in this regard [12]. Todays, various methods like TLC and HPLC are used to identify and measure the level of sterigmatocystin in crops, cattle feed and foodstuffs [7, 13]. This study aims to examine the effect of *aflD(nor-1),aflO(omtB)* and *aflJ(estA)* on sterigmatocystin, as an ultimate precursor aflatoxin, in the samples of cattle feed collected form 21 industrial animal husbandary in Tehran and Alborz provinces.To do so, these samples were isolated and cultured based on macroscopic and microscopic methods, and then PCR technique was also used to detect the isolated *Aspergillus* fungi. To identify the relationship between aflatoxin genes with sterigmatocystin, 20 samples of cattle feed, containing one, two or three positive aflatoxin genes were randomly selected, examined under the ultraviolet light and finally, evaluated using TLC.

MATERIALS AND METHODS

Sample collection

121 samples of corn, internal and imported barely, wheat bran and soybean meal, each weighing 200-250 gr,were collected from 21 silos and warehouses of industrial animal husbandry in Alborz and Tehran provinces during summer and winter, 2014-2015. These samples were put into the sterile paper pocket, coded, sent to the laboratory and kept below 20˚C.

Aspergillus Isolation and Identification of cattle feed

To isolate *Aspergillus* fungi from cattle feed, 5 mm distilled water was added to the samples of cattle feed put in test tubes and after two hours, the supernatant liquid was removed, vortexed and centrifuged. Hence, 200 microliter of the resulting mixture is transferred to sabouraud dextrose agar (SDA), containing chloramphenicol by a sterile micropipette, and then plates were maintained for a week in the dark at+25˚C.The grown isolates obtained from each plates were undergone macroscopic and microscopic examinations using Klich classification keys(Klich and Pitt 1988; Klich 2002). Colonies enjoying genus *Aspergillus* characteristics were transferred to yeast extract sucrose agar (YES) medium and purified there using liner culture method(Samson et al. 2004).

Molecular Characterization

Detection of Aflatoxin Genes aflD(nor-1),aflO(omtB) and aflJ(estA)

The biosynthesis pathway of aflatoxin gene cluster proposed. All *Aspergillus* isolates were examined in the presence of *aflD(nor-1),aflO(omtB)*and *aflJ(estA)* the results were presented in Table 1.

Table 1. Aflatoxin biosynthetic genes and functions

Old name	New name	Enzyme/product	Function in the pathway
nor1	aflD	NOR reductase	norsolorinic acid (NOR) → averantin (AVN)
omtB	aflO	O-methyltransferase B	DHDMST (dihydrodemethylsterigmatocystin) → DHST (dihydrosterigmatocystin)
estA	aflJ	Esterase	versicona hemiacetal acetate (VHA) → versiconal (VAL)
Clustered pathway genes in aflatoxin biosynthesis [14-16].			

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 to extract DNA.500 microliters lysis buffer (containing 1

mollar Tris-HCI (PH=8), 0.5 molar EDTA (Ph=8) and 7.45 g KCI), a pile of about 60 gr mycelium was added from *Aspergillus* colony and then crushed by hand or 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 microtub and centrifuged for 10 minutes at 12000 g. The supernatant liquid was discarded and about 0.8 microliter 70degree 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 DNA was mixed with distilled water by gently tapping. The resulting liquid is frozen and stored at -20˚C as a pure DNA solution.

PCR amplification

To investigate the molecular characteristics of the *Aspergillus* of cattle feed, *ITS1-5.8s-ITS2* gene fragments and *aflD, aflO* and *aflJ* were used(Scherm et al. 2005). Primers were designed using OLIGO7 software based on standard sequences in the gene bank. Additionally, BLAST software was employed to confirm that the primers were novel (Table 2). 5 microliters of extracted DNA, 1 microliter of forward and reverse primers, 10 microliters of PCR master mix of Ampliqon Company (containing 0.2 U/µl of Taq DNA polymerase, 0.4 milimolar of dATP and dNTP (dTTP, dCTP, dGTP and 3 milimolar of MgCl2) and enough sterile deionized distilled water (ddH2O) were added up to 20 microliters. PCR is implemented as presented in

Table **3**.

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

Primer code	Target gene	Primer sequences	PCR product size (bp)	Accession no
ITS-1for	ITS	5'- GGCTTTGTCACCCGCTCTGT -3'	691	AF027863.1
ITS-2rev		5'- ACGACCATTATGCCAGCGTCC -3'		
AflD-1for	aflD(nor-1)□	5'- CTCATCACACGCAGGCATCGG -3'	702	FN398169.1
AflD-2rev		5'- AGATGCCTGCCACACTGTCT -3'		
AflO-1for	aflO(omtB)□	5'- TTACGATTTGATGGAGCAGG -3'	358	HM355030.1
AflO-2rev		5'- AGGTTCTCTTGGCTACAG -3'		
AflJ-1for	aflJ(estA)□	5'- CCTGACCATCTCCGACCCGTTC -3'	1145	Xm-002379904.1
AflJ-2rev		5'- AACTTCCCGCATCACCACGAG -3'		
□ Aflatoxin biosynthetic genes are named as proposed by (Yu et al. 2004), old names are reported in brackets.				

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
ITS	95 ◦C	2 min	95 ◦C	30 sec	62 ◦C	45 sec	72 ◦C	45 sec	72◦C	7 min
aflD	95 ◦C	3 min	95 ◦C	30 sec	61.4◦C	40 sec	72◦C	30 sec	72◦C	7 min
aflO	95 ◦C	3 min	95 ◦C	30 sec	52.4◦C	30 sec	72◦C	30 sec	72◦C	7 min
aflJ	95 ◦C	3 min	95 ◦C	30 sec	59 ◦C	45 sec	72◦C	1:30 min	72◦C	7min

Sterigmatocystinof the Isolates

Fluorescence on Yeast Extract Sucrose Agar (YES)

To examine the ability of sterigmatocystin production in the *Aspergillus* isolates of the samples, those speciess, in which aflatoxin genes *aflD, aflO* or *aflJ*, or all of them were positive were cultured on YES, stored for 10 days in the dark at +25˚C and finally evaluated under the ultraviolet light(365 nanometer) [17].

Sterigmatocystin Analysis by TLC

Four samples with toxic *Aspergillus* isolates and positive aflatoxin genes *aflD, aflO* and *aflJ* were randomly selected from each cattle feed (A: corn, B1: Iranian Barely, B2: imported barely, D: wheat bran and E: soybean meal), cultured on YES medium and stored for 10 days in the dark at 25˚C. To investigate the sterigmatocystin production in the isolates, TLC technique was used. Accordingly, to extract sterigmatocystin from collected samples, 500 ul of samples were mixed with 250 ul of chloroform, were pipetted for five minutes and dotted onto a silica gel TLC plate so that the distance of the dots form the lower margin of TLC plate was about 2 cm, and they were dried at 25˚C. Then, 50 ml extraction solvent (including 40 ml toluene, 7.5 ml methanol and 2.5 ml acetic acid) was added to

solvent tank so that the depth of solvent at the bottom of the tank should be less than 2 cm. TLC plate was put in the tank so that the solvent stays 4 to 5 cm above the dotted line (about 15 to 20 minutes) and then, TLC was extracted from the solvent tank, dried and finally observed under the ultraviolet light (365 nanometer).

RESULTS

Morphologic and microscopic characteristics of Aspregillus in the cattle feed

A total of 121 samples of cattle feed collected from industrial animal husbandry were cultured and examined. After a week, four *Aspergillus* species, mainly *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus nidulans* and *Aspergillus nomius* were isolated on YES [18]. To do so, the form and color of the surface and back of colonies on plates, the structure of mycelium, conidiofor and phyalids, the form, size and color of spores and their accumulation were examined using mycological keys and reliable resources [19, 20].

Figure 1. Samples of cattle feed were cultured on SDA for a week at 25˚C

Figure 2. Grown isolates in each plate containing YES were cultured for a week at 25˚C.

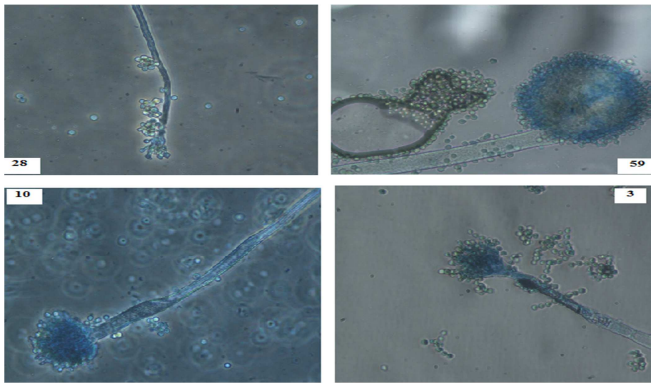

Figure 3. Microscopic photo of Aspergillus samples in cattle feed at 40x magnification

Molecular identification of Aspergillus isolates and Sterigmatocystin Production

In this study, 121 samples of cattle feed including A: corn, B1: Iranian barely, B2: imported barely, D: bran and E: soybean meal, are randomly selected from industrial animal husbandry in Alborz and Tehran provinces. To isolate *Aspergillus* fungi, *ITS* general gene fragment is used, and to detect the aflatoxigenic species, aflatoxin genes *(aflD, aflOand aflJ)* available in the cattle feeds are considered based on PCR technique. It is noteworthy that two standard

samples, namely *Aspergillus flavus 5004* and *Aspergillus parasiticus 5018* were employed as positive controls throughout the examination. The results indicate that 67 samples, i.e. 55.37% show *Aspergillus* contamination. Accordingly, 25 samples involve *aflD*, 61 samples have *aflO*and 23 samples is made up of *aflJ*.

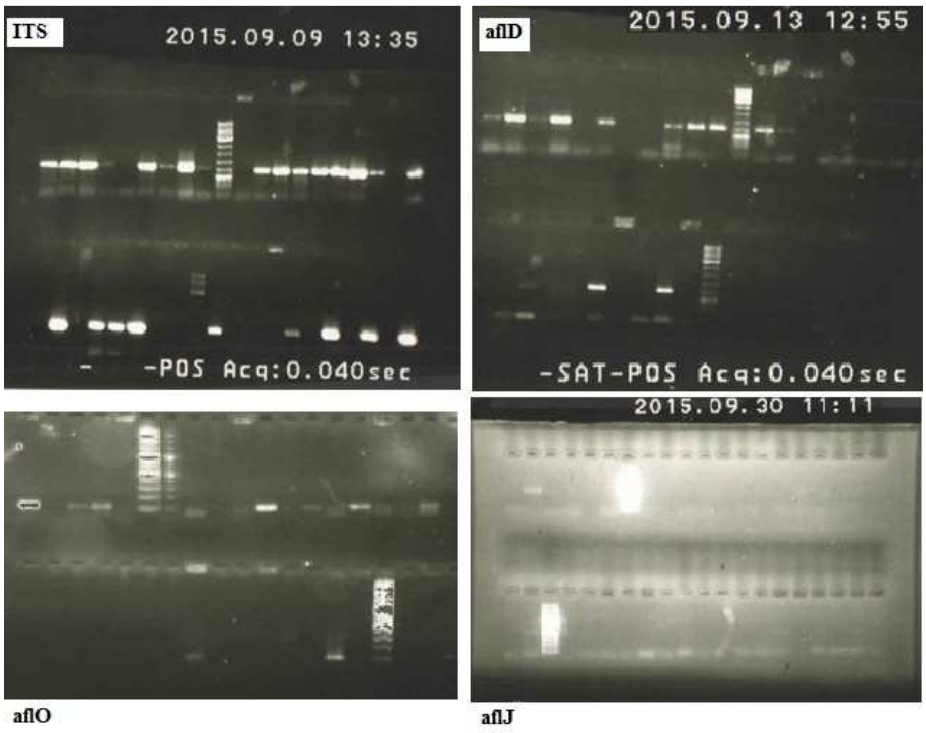

Figure 4. The images of gel electrophoresis of PCR products for expression of genes (ITS, aflD, aflO and aflJ) of Aspergillus isolated from cattle feed in 1% agarose gel and ladder 1kb

To identify the relationship between aflatoxin genes with sterigmatocystin, 20 samples of cattle feed, containing one, two or three positive aflatoxin genes were randomly selected, examined under the ultraviolet light and finally evaluated using TLC (

Figure 5and Table 4).

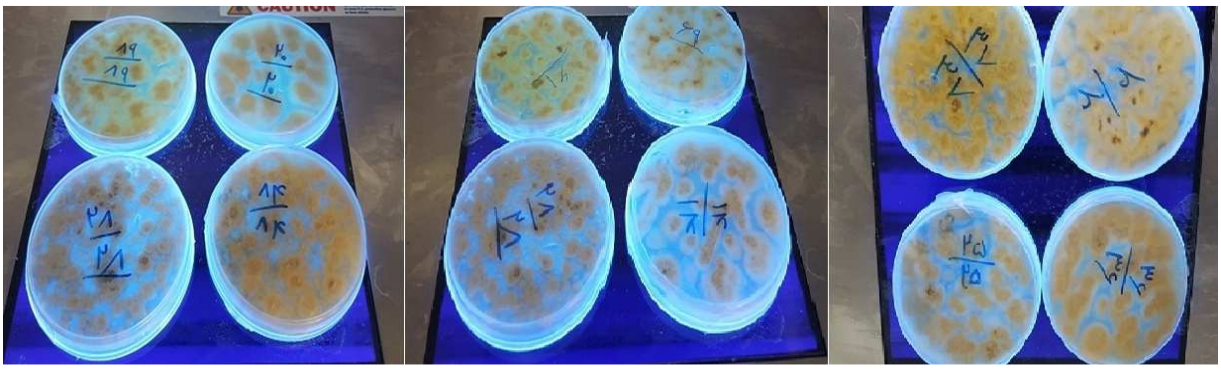

Figure 5. Colonies of aflatoxigenicspeciess of Aspergillus are observed under the ultraviolet light (356 nm) as various types of green, blue and white halos, generating different types of aflatoxins while non-aflatoxigenicspeciess were observed with no halo[17]

Table 4. The PCR products of aflatoxin genes (aflO, aflD and aflJ) are tested. Black box represents expressed gene and gray box denotes no expressed gene.The presence of STC was examined using TLC method, in which (+) denotes high level of STC and (×) denotes low level of STC in the samples. (F) stands for the standard Aspergillus flavus and (P) stands for the standard Aspergillus parasiticus

Sample Type	*Number*	*Isolate*	*STC*	*ITS*	*AFLD*	*AFLO*	*AFLJ*	*Sample Type*	*Number*	*Isolate*	*STC*	*ITS*	*AFLD*	*AFLO*	*AFLJ*
1:A	1			_				5:E	34	*A. flavus*	×	+	+	+	_
1:B2	2	*A. flavus*		+	+	+	_	6:A	35			_			
1:B2	3	*A. flavus*		+	+	+	_	6:B1	36			_			
1:B2	4	*A. nomius*		+	+	+	_	6:D	37	*A. nidulans*		+	_	_	_
1:B2	5			_				6:E	38	*A. flavus*		+	+	+	_
1:B2	6	*A. flavus*	×	+	+	+	+	7:A	39	*A. flavus*	×	+	+	+	+
1:C	7			_				7:A	40	*A. flavus*		+	_	+	_
1:D	8	*A. nidulans*		+	_	+	_	7:B1	41	*A. nidulans*		+	_	+	_
1:E	9	*A. flavus*	×	+	+	+	_	7:B1	42			_			
1:E	10	*A. nomius*		+	+	_	_	7:D	43	*A. flavus*		+	_	+	_
2:A	11			_				7:E	44			_			
2:B1	12	*A.parasiticus*		+	+	+	_	8:A	45			_			
2:B1	13	*A. flavus*	×	+	+	+	+	8:B1	46	*A. flavus*		+	_	+	_
2:B2	14	*A. flavus*		+	_	_	_	8:D	47	*A.parasiticus*		+	_	+	_
2:D	15			_				8:E	48			_			
2:E	16	*A. flavus*		+	_	+	+	9:A	49	*A. flavus*		+	_	+	_
2:E	17	*A. nidulans*	+	+	+	+	+	9:B2	50	*A. nidulans*		+	_	+	_
2:E	18	*A. nidulans*		+	_	+	_	9:B2	51	*A. flavus*	×	+	_	+	_
3:A	19			_				9:D	52	*A. flavus*	×	+	+	+	+
3:B2	20	*A. flavus*		+	_	+	+	9:D	53	*A. flavus*		+	_	_	+
3:D	21			_				9:D	54			_			
3:E	22	*A. flavus*		+	_	+	_	9:D	55	*A. flavus*		+	+	+	_
4:A	23	*A. flavus*		+	_	+	_	9:E	56	*A.parasiticus*	×	+	+	+	_
4:A	24	*A. flavus*	×	+	_	+	+	10:A	57			_			
4:B2	25	*A.parasiticus*		+	_	+	_	10:A	58	*A. nomius*		+	_	+	_
4:D	26	*A. nidulans*		+	_	+	+	10:B1	59	*A. flavus*	×	+	+	+	_
4:D	27			_				10:B2	60			_			
4:E	28			_				10:B2	61	*A.parasiticus*		+	+	+	_
5:A	29			_				10:D	62			_			
5:B2	30			_				10:E	63	*A. flavus*		+	_	+	+
5:B2	31	*A.parasiticus*		+	_	+	_	11:A	64	*A. flavus*		+	_	+	_
5:B2	32	*A. flavus*		+	_	+	+	11:A	65			_			
5:D	33	*A. flavus*	×	+	+	+	_	11:B1+B2	66			_			

Sample Type	*Number*	*Isolate*	*STC*	*ITS*	*AFLD*	*AFLO*	*AFLJ*	*Sample Type*	*Number*	*Isolate*	*STC*	*ITS*	*AFLD*	*AFLO*	*AFLJ*
11:D	67	*A. nomius*		+	_	+	+	17:E	100			_			
11:E	68	*A. flavus*		+	_	+	+	18:A	101	*A.parasiticus*		+	+	+	+
11:E	69	*A. nidulans*		+	_	_	+	18:B2	102			_			
12:A	70			_				18:D	103			_			
12:B1	71	*A. flavus*	×	+	_	+	_	18:E	104			_			
12:B2	72			_				19:A	105			_			
12:D	73			_				19:A	106			_			
12:E	74			_				19:B2	107	*A. flavus*		+	_	+	+
13:A	75			_				19:B2	108	*A.parasiticus*		+	+	+	_
13:B1	76	*A. flavus*		+	_	+	_	19:B2	109			_			
13:D	77	*A. flavus*		+	_	+	+	19:D	110	*A. flavus*	×	+	_	+	+
13:E	78			_				19:E	111			_			
14:A	79	*A. flavus*		+	_	+	_	20:A	112	*A. nidulans*	+	+	+	+	+
14:B1+C	80	*A.parasiticus*		+	_	+	_	20:A	113	*A. flavus*	×	+	+	+	_

14:D	81			_				20:A	114	*A. flavus*		+	_	_	_
14:E	82			_				20:B2	115			_			
15:A	83	*A. flavus*		+	_	+	+	20:D	116	*A. flavus*		+	_	+	_
15:A	84			_				20:E	117	*A. nidulans*		+	_	+	_
15:B2	85	*A. nidulans*		+	_	+	+	21:A	118			_			
15:B2	86	*A. flavus*	×	+	+	+	+	21:B2	119	*A.parasiticus*	×	+	+	+	_
15:D	87	*A. flavus*	×	+	+	+	_	21:D	120			_			
15:D	88	*A.parasiticus*		+	_	+	+	21:E	121			_			
15:E	89			_				*F*	*ST*	*A. flavus*	×	+	+	+	+
16:A	90			_				*P*	*ST*	*A.parasiticus*	×	+	+	+	+
16:B1	91			_											
16:B2	92			_											
16:C	93			_											
16:D	94			_											
16:E	95			_											
17:A	96			_											
17:B1	97	*A.parasiticus*	×	+	_	+	_								
17:C	98			_											
17:D	99			_											

DISCUSSION

Todays, mycotoxin-producing fungi are scattered worldwide and three genera of fungi ,namely, *Aspergillus*, *Penicillium* and *Fusarium* are recognized as the major mycotoxin-producing fungi [1]. More than 20 species of *Aspergillus* are recognized to produce STC as a final product [21-24]. This compound is amongst the final aflatoxin precursor in toxigenic fungi, and genetic-molecular methods have revealed that coding genes STC in *Aspergillus nidulans* act like those in *Aspergillus flavus* and *Aspergillus parasiticus* [25]. Sterigmatocystin is a metabolite, structurally similar to aflatoxin [26]. *Aspergillus flavusSclerotia* and *Aspergillus minisclerotia* are amongst the *Aspergillus section Flavi* producing STC. Moreover, *Aspergillus emericella, Aspergillus ochraceus, Aspergillus rambelli, Aspergillusnidulans* species and *Aspergillus versicolor* species also produce this toxin [27].

TLC and HPLC are common methods used to identify STC [28]. This study uses TLC because of the fastexamination of the samples, its economic advantages and the inability of HPLC in UV or florescence absorption [13, 29, 30]. This research aims to reveal and confirm the relationship between aflatoxin genes and STC in the cattle feed using PCR and TLC methods, respectively [31]. The microscopic studies of the cattle feeds indicate that four species of *Aspergillus*, namely *Aspergillus flavus, Aspergillus parasitic*, *Aspergillus nidulans* and *Aspergillus nomius* have grown on these compounds and contaminated them.

Fenteet alexamined the fluorescence characteristic of 14 isolates of *Aspergillus* species in Aflatoxin Producing Ability (APA) medium. Among the examined isolates, seven *Aspergillusparasiticus* isolates and five *Aspergillusflavus* isolates enjoyed this characteristic. This experiment was reconducted in YES medium using HPLC and the same results were drawn, confirming the inability of some isolates of *Aspergillus* fungi in producing aflatoxin(Fente et al. 2001).Sales et al studied 78 samples of cattle feed in Thailand and Vietnam and reported 78% of the collected samples were contaminated with *Aspergillus parasiticus* and *Aspergillusflavus* [32]. Halt also examined wheat, barley and corn used as cattle feed in Croatia and found that *Aspergillusflavus* is the main cause of contamination [33]. Studying 258 samples of corn based on PCR method in Kenya, Sheila Okoth et al used aflatoxin genes *aflD* and *aflQ* to detect aflatoxigenic species of *Aspergillusflavus* and *Aspergillus parasiticus*. They found that *Aspergillus* section *Flavi*, as aflatoxigenic *Aspergillus*, accounts for the most portion of contamination [34]. In the next stage, PCR was used to identify the *Aspergillus* isolated from cattle feed. To do so, *ITS* was designed to isolate *Aspergillus* fungi in 691 bp band using PCR, and aflatoxin genes like *aflD* with 702 bp band, *aflO* with 358 bp band and*aflJ* with 1145 bp band were isolated using PCR [35]. The results of this study indicated that 55.37% of 121 samples were contaminated with *Aspergillus*. Accordingly, among these isolated samples, 37.31% has *aflD*, 90.01% has *aflO* and 34.32% has *aflJ*, among which 20 randomly selected samples, containing one, two or three positive aflatoxin genes, were examined under the ultraviolet light and finally, observed as toxigenitic using TLC. The results of TLC suggested that STC, which is the ultimate precursor of aflatoxin, is produced if all three aflatoxin genes are positive. It is also believed that sterigmatocystin production is directly correlated with *aflD,aflO* and *aflJ*.

The aflatoxigenic characteristics of *Aspergillus* have drawn a lot of attention. Accordingly, corn sample No 112(20:A) and soybean meal sample No 17 (2:E), which have three positive aflatoxin genes, produced high level of sterigmatocystin, and microscopic and macroscopic examinations indicated that their characteristics were similar to those of *Aspergillus nidulans*. TLC showed that the band of these two samples were sharp, indicating low level of sterigmatocystin in the cattle feed, particularly in the samples. However, since sterigmatocystin is a dangerous a and may enter animal and human feed and food, standard regulations for using permitted limit of this toxin should be taken into account in Iran and other developing countries. This may affect the internal cattle feeds as well asthose imported from other countries. According to the reports, most of the imported cattle feeds are contaminated with aflatoxigenic *Aspergillus*. Therefore, it is necessary to consider the toxicity of the isolates to prevent likely future problems [4, 10, 11].

CONCLUSION

As can be seen, aflatoxigenic *Aspergillus* accounts for most portion of contamination in cattle feed. Therefore, controlling cattle feeds, aeration of silos and warehouses and training ranchers to store and maintain cattle feed appropriately can inhibit the growth of aflatoxigenic fungi and prevent mycotoxin from entering the human and animal health cycle. To generalize the results of this study for the rest of the country, sampling in other provinces is suggested.

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