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Evaluation of the Cattle Ration Feed Contamination Amount with the *Aspergillus* fungi by PCR-based Technique and Based on *ITS* Gene Sequences

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Aflatoxin is carcinogenic metabolites that produced by a number of species *Aspergillus* section Flavi. Cattle feed contaminated with Aflatoxin endanger human and animal health. The aim of this study is to determine amount of contamination to the *Aspergillus* section Flavi in five main animal feed with PCR molecular technique based on *ITS* regions and fungal colony. In this study, 121 ration samples were used from 21 industrial animal husbandry warehouses and silage in Tehran and Alborz Province. After isolating and culture in specific sphere of yeast extract sucrose agar (YESA), isolated *Aspergillus* fungi were studied by macroscopic and microscopic method. For molecular identification of the *Aspergillus* fungi from PCR method, *ITS* sequencing also was used and finally grown colonies were counted to determine an amount of the fungal contamination. The results showed that 67 samples from 121 ones were positive in which represents the 55/37% contamination amount to the *Aspergillus* fungi, and also the highest level of fungal contamination in imported barley, wheat bran, soybean meal and corn is about 14/16%, 12/5%, 10/83% and 10/83% respectively, while the lowest fungal contamination belongs to the internal barley with 7/5%. Therefore, there is the need to develop a simple, rapid and sensitive method for the detection of *Aspergillus* fungi. With regard to the high fungal contamination of imported cattle feed while its amount is very low in internal products, it is better that officials try to increase internal production as well as more controlling over the production, transport and import of cattle feed.

Keywords: *Aspergillus* fungi, Aflatoxin, PCR, *ITS*, Cattle feed.

Mycotoxins are produced highly in cereal grains such as corn, sorghum, barley, wheat, cottonseed, peanut meal and fodder, before and during the harvest in humidity conditions (Richard *et al*. 2003). The Mycotoxin term first was introduced in 1962, after depth of one hundred thousand turkeys in Britain for an unknown disease. The disease caused by a toxin produced by the *Aspergillus flavus* fungi in birds feed (Wogan and Pong 1970). Following the crisis, scientists suspect other fungal metabolites may also be fatal. From 1960 to 1975 was the golden era of Mycotoxins studies, as scientists have done extensive research on these toxigenic factors (Bennett and Klich 2003). Mycotoxins are compounds produced by some strains of different fungal species especially *Aspergillus*, and also are dangerous for the health of humans and animals

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(Humans *et al*. 2002). *Aspergillus* genus has been classified in different categories based on morphological characteristics and genomic sequences which in the meantime *Aspergillus* section Flavi, *Circumdati* genus threatens public health by producing mycotoxins, particularly aflatoxin B1 which is known as the strongest natural hepatocarcinogen . They produce aflatoxin and can cause tissue necrosis, cirrhosis and liver cancer (Richard *et al*. 2003; Chu 1991). Strains of *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* are the most important species of this category (Yu *et al*. 2004; Varga *et al*. 2009; Ehrlich *et al*. 2003; Yu *et al*. 2000). Identification of *Aspergillus* fungi from various aspects such as pathogenicity, virulence and industrial is important in terms to whether be aflatoxin productive or unproductive (Bennett *et al*. 2007; Murphy *et al*. 2006). In order to distinguish these species, typically morphological characteristics are used such as macroscopic traits like color and colony growth rate, and also microscopic traits like morphology and measurement of Vesicle, Conidia, Conidiophore, Phialide and Metula (Klich 2002; Klich and Pitt 1988). *Aspergillus* species have very similarities to each other in order to morphological traits and on the other hand, this method is expensive, time-consuming and finally also does not have a high degree of accuracy. Therefore, molecular techniques, such as PCR are used to investigate the presence or absence of a gene, is faster and more accurate detection of aflatoxin-productive *Aspergillus* fungi. The Real-Time PCR is another method which is automated, rapid, repeatable and highly accurate technique to differ aflatoxin productive fungal samples from unproductive ones (Geisen 1996; Shapira *et al*. 1996; Färber *et al*. 1997; Sweeney *et al*. 2000; Criseo *et al*. 2001). These techniques are used as proprietary methods and the final diagnosis, and microarray technology will be of great worldwide importance in the near future.

The aim of this study is to isolate contaminated samples to *Aspergillus* fungi with morphological and microscopy characteristics, and PCR molecular technique based on *ITS* region. Then investigate the contamination of *Aspergillus* fungi in five main cattle feed rations in Tehran and Alborz province industrial animal husbandry.

MATERIALS AND METHODS

Samples collection

121 samples were collected randomly from 21 industrial animal husbandry warehouses and silos in Tehran and Alborz province, each in the amount of 250-500 grams, including corn (A), internal barley (B1), imported barley (B2), wheat bran (D) and soybean meal (E). Sampling time was 2014 - 2016 summer and winter respectively. These samples were placed in sterile bags and after encoding, (the name of animal husbandry, cattle feed type, date of collection) they were transferred to the laboratory. Samples were stored at -20 ° C until were been used.

Isolation *Aspergillus* fungi from Cattle feed rations

First, for isolating *Aspergillus* fungi from cattle feed, 5 ml of distilled water was added to each cattle feed sample test tubes and after 2 hours, the supernatant liquid was collected; after vortex and centrifugation, 200 microliters of the mixture that obtained by sterile micropipette in saboro dextrose agar culture containing chloramphenicol, (to prevent the growth of bacteria and yeasts) was transferred; the plates was kept in the dark place at a temperature of 25 ° C for a week (Samson *et al*. 2004). Then grown isolated mixtures in each plate were examined macroscopically and microscopically (Klich and Pitt 1988). Colonies that showed *Aspergillus* characteristics, were purified and transferred to yeast extract sucrose agar (YESA) by using methods in which culturing in slope within the tube.

DNA Extraction

For DNA extraction, a few microliters of stored spore suspension of *Aspergillus* isolates was transferred to the plate containing YESA and the colony were used for DNA isolation for a week. 500 microliters of buffer (containing 1 M Tris-HCL (pH = 8), 0.5 M EDTA (pH = 8), 7.45 g KCl), 60 mg of fungal myceliums was added to *Aspergillus* colonies; they were crushed by vortex and manual method for 45 seconds and then centrifuged for 10 min with 5000 RPM. The supernatant liquid was transferred to a new tube and 300 microliters of cold isopropanol (which is kept at -20 ° C) was added; cell lysis and isopropanol were mixed by shaking micro-tubes, and then was centrifuged for

10 minutes with 12000 RPM. This time the supernatant liquid was removed and about 0.8 microliters of 70% alcohol added to the sediment and after 15 min we put it in the incubator at 37 ° C to evaporate remaining alcohol; finally, 50 microliters of deionized distilled water was added to the sediment and DNA dissolved with distilled water by gently tapping. The obtained liquid as pure DNA solution was kept in freezer at -20 ° C until required for use.

PCR Amplification

For molecular identification of cattle feed rations *Aspergillus, ITS* gene parts were used in this study and by using OLIGO7 powerful software, primers were designed based on standard sequences in the gene bank (Pryce *et al*. 2003). Proper application of the primers was determined by using the BLAST software. So based on these parts, *ITS1-5.8-ITS2* general primers were made by Macrogene company. 5 microliters of extracted DNA (Table 1), 1 microliters of each of the Forward and Reverse primers, 10 microliters of master-mix PCR of Amplicon company [which contains 0.2 units per microliter of Taq DNA polymerase, 0.4 mM of each (dATP, dTTP, dCTP, dGTP) dNTP and 3 mM $MgCl_2$] and the necessary amount of sterile deionized distilled water (ddH_2O) was added to reach final volume of 20 microliters. In this study, the prepared master mix of Amplicon Company was used. Finally, the materials mixed slowly and were placed inside the thermo cycler device. All the steps were done under sterile conditions, laminar hood and within the ice dish. In the next step, for strengthen the gene in PCR reactions, genomic of isolated *Aspergillus* species was done with thermal model by using Forward, Reverse and DNA primers. PCR product with markers by bp100 molecular weight was electrophoresed on 1% Agarose gel containing *Ethidium* bromide, and it was studied by using documentation gel device. The PCR thermal program was performed according to Table 2.

RESULTS

Morphologic and microscopic characteristics of *Aspergillus* fungi in Cattle Feed

A total of 121 samples of cattle feed collected from industrial animal husbandry were cultured and examined. After a week, *Aspergillus* species were isolated on YES (Samson *et al*. 2004). 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 (Klich and Pitt 1988; Peterson 2003).

Molecular identification of isolated *Aspergillus* fungi by PCR method

121 samples that collected by using the PCR method, were studied to identify *Aspergillus* fungi. After DNA extraction, the DNA concentration was measured by using NANO drop spectrophotometer. The amount of DNA was homogenized in all samples until get to 100 ngr. As noted above, 121 samples were randomly selected which is contained corn (A), internal barley (B1), imported barley (B2), wheat bran (D) and soybean meal (E). *ITS* general gene parts were used to isolate *Aspergillus* fungi in which the results of PCR products showed the 691 bp band development, and revealed that 67 of tested samples from 121 belongs to the *Aspergillus* genus which indicative 55.37% of *Aspergillus* fungi contamination. It is noteworthy that two standard samples (5004 *Aspergillus flavus* and 5018 *Aspergillus parasiticus*) were present at all stages for positive controlling (Figure 3 and Table 3).

Counting the fungal colonies in cattle feed rations and estimating the amount of contamination

The abundance of fungal contamination in corn samples of cattle feed was found when different analysis and isolation of different isolates were done; 29 fungal colonies out of grown corn isolated samples were grew up of which 13 colonies were related to *Aspergillus*. It can be said that the amount of corn contamination with *Aspergillus* is about 10.83 percent out of total grown isolates level. The percentage is our own expectations as well because corn fungal contamination is caused by being imported from tropical countries, transferring in hot and humid environment and improper storage conditions in shipping warehouses. Therefore, this issue requires special attention to internal production and also monitoring imports as well.

Also the abundance of fungal contamination in internal barley samples of cattle feed was found when different analysis and isolation of different isolates were done; 13 fungal

colonies grew out of grown internal barley isolates sample of which 9 colonies were related to *Aspergillus*. Compared to the total amount of grown fungal isolates, it can be said that the internal barley contamination is about 7.5 percent out of total *Aspergillus* fungi contamination.

The abundance of fungal contamination in imported barley samples of cattle feed was found when different analysis and isolation of different isolates were done; 26 fungal colonies grew out of grown imported barley isolates sample of which 17 colonies were related to *Aspergillus*. Compared to the total amount of grown fungal isolates, it can be said that the imported barley contamination is about 14.16 percent out of total *Aspergillus* fungi contamination.

The abundance of fungal contamination in wheat bran samples of cattle feed was found when different analysis and isolation of different isolates were done; 26 fungal colonies grew out of grown wheat bran isolates sample of which 15 colonies were related to *Aspergillus*. Compared to the total amount of grown fungal isolates, it can be said that the wheat bran contamination is about 12.5 percent out of total *Aspergillus* fungi contamination.

Finally, the abundance of fungal contamination in soybean samples of cattle feed was found when different analysis and isolation of different isolates were done; 25 fungal colonies grew out of grown soybean isolates sample of which 13 colonies were related to *Aspergillus*. Compared to the total amount of grown fungal isolates, it can be said that the soybean contamination is about 10.83 percent out of total *Aspergillus* fungi contamination.

Table 1. Sequences of the nucleotide primers used in this study

Primer code	Target gene	Primer sequences	PCR product size (bp)	Accession no
ITS-1for ITS-2rev	ITS	5'- GGCTTTGTCACCCGCTCTGT -3' 5'- ACGACCATTATGCCAGCGTCC -3'	691	AF027863.1

[a]Aflatoxin biosynthetic genes are named as proposed by (Yu et al. 2004), old names are reported in brackets

Table 2. Heat program used for PCR

PCR steps	1 cycle		34 cycle						1 cycle	
	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

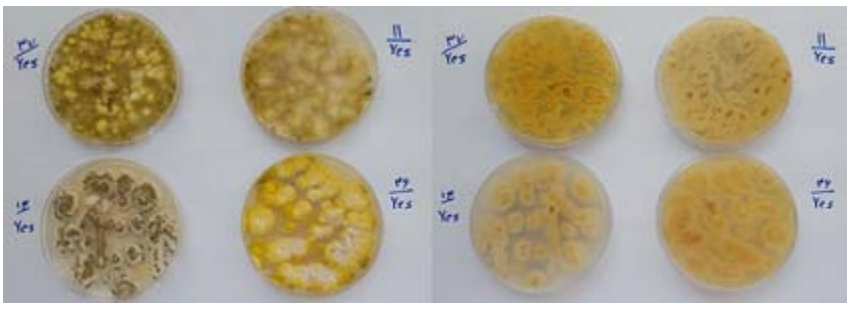

Fig. 1. Grown isolates of *Aspergillus* fungi in each plate containing YESA were cultured for a week at 25°C

DISCUSSION

Aflatoxins are fungi secondary metabolites and mainly produce by *Aspergillus* section Flavi including *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* (Varga *et al*. 2009; Frisvad *et al*. 2005). Today, Mycotoxin productive fungal are world-wide distribution, and include more than 10 species of the genus *Aspergillus* that is often in Flavi section. The optimum temperature for producing aflatoxin has been reported between 24 -30 ° C (Ogundero 1987; Sorenson *et al*. 1967). In this study, we decided to survey the contamination of *Aspergillus* fungi in Iran through morphological and microscopic and molecular techniques such as PCR and growth colonies numeration (Gilbert and Vargas 2003). Macroscopic and microscopic studies of cattle feed rations showed that several species of *Aspergillus* have grew on these rations, and have contaminated them, that *Aspergillus flavus*, *Aspergillus parasiticus* were among them (Sales and Yoshizawa 2005). In the next phase, the PCR reaction was used for molecular detection of isolated *Aspergillus* of cattle feed . The universal *ITS* gene part was designed for this purpose and fungal associated with genus *Aspergillus* by 691 bp band was separated in the PCR reaction (Pryce *et al*. 2003; Rahimi *et al*. 2016a). The results of this study showed that based on the information, from 121 tested samples of cattle feed rations, 55.37 percent of all have contamination with the *Aspergillus* fungi (Scherm *et al*. 2005; RAHIMI *et al*. 2016b).

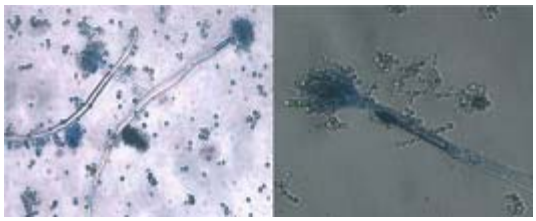

Fig. 2. Microscopic photo of *Aspergillus* samples in cattle feed at 40x magnification

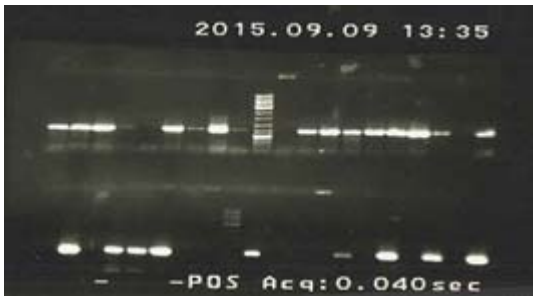

Fig. 3. The images of gel electrophoresis of PCR products for *ITS* gene expression of *Aspergillus* isolated from cattle feed ration in 1% Agarose gel alongside Ladder 1k

Sales *et al* (2005) studied 78 cattle feed samples from Thailand and Vietnam, and then they reported 94% contamination of samples by *A. parasiticus* and *A. flavus* species (Sales and Yoshizawa 2005). Halt also examined wheat, barley and corn used as cattle feed in Croatia and found that *Aspergillus flavus* is the main cause of contamination (Halt 1994). Scherm *et al* in 2005 introduced aflatoxin as a secondary metabolite which produced by *Aspergillus* section Flavi, especially *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* (Scherm *et al*. 2005). Sheila Okoth and colleagues (2012) studied 258 corn samples in Kenya by using the PCR method; they used genes *aflQ* and *aflD* aflatoxin productive gen to identify *Aspergillus flavus* and *Aspergillus parasiticus* toxin species, and attributed the maximum amount of contamination to *Aspergillus* section Flavi. They reported that the highest and the lowest levels are belonging to aflatoxins toxins B and G (Okoth *et al*. 2012). Villa and markesi in 2009 were investigated B1 aflatoxin contamination in 55 samples of wheat by using HPLC and immunoaffinity column. They declared that 3/56% of B1 aflatoxin was contaminated with amount of 1.42 ng/g (Villa and Markaki 2009).

In this study, the *Aspergillus* section Flavi fungi contamination of cattle feed were determined within the scope of contamination in food products which represents the accommodation of the results to previous researches. The results showed that the imported barley and wheat bran have the highest amount of *Aspergillus* contamination by 16/14% and 12.5% respectively, and the lowest was related to the internal barley with the 5/7%. According to the World Food and Agriculture Organization reports, FAO (Food and Drug Administration) annually 20 percent of food products are contaminated with fungal toxins in the world in which the contamination caused by aflatoxin has greater level (Van Egmond *et al*. 2007). The FAO announced that the maximum permitted

Table 3. The PCR products of *ITS* genes are tested. Black box represents expressed gene and gray box denotes no expressed gene .(F) stands for the standard *Aspergillus flavus* and (P) stands for the standard *Aspergillus parasiticus*

Sample Type	No	Isolate	ITS	Sample Type	No.	Isolate	ITS	Sample Type	No.	Isolate	ITS	Sample Type	No.	Isolate	ITS
1:A	1		_	5:E	34	*A. flavus*	+	11:D	67	*A. nomius*	+	17:E	100		_
1:B2	2	*A. flavus*	+	6:A	35		_	11:E	68	*A. flavus*	+	18:A	101	*A.parasiticus*	+
1:B2	3	*A. flavus*	+	6:B1	36		_	11:E	69	*A. nidulans*	+	18:B2	102		_
1:B2	4	*A. nomius*	+	6:D	37	*A. nidulans*	+	12:A	70		_	18:D	103		_
1:B2	5		_	6:E	38	*A. flavus*	+	12:B1	71	*A. flavus*	+	18:E	104		_
1:B2	6	*A. flavus*	+	7:A	39	*A. flavus*	+	12:B2	72		_	19:A	105		_
1:C	7		_	7:A	40	*A. flavus*	+	12:D	73		_	19:A	106		_
1:D	8	*A. nidulans*	+	7:B1	41	*A. nidulans*	+	12:E	74		_	19:B2	107	*A. flavus*	+
1:E	9	*A. flavus*	+	7:B1	42		_	13:A	75		_	19:B2	108	*A.parasiticus*	+
1:E	10	*A. nomius*	+	7:D	43	*A. flavus*	+	13:B1	76	*A. flavus*	+	19:B2	109		_
2:A	11		_	7:E	44		_	13:D	77	*A. flavus*	+	19:D	110	*A. flavus*	+
2:B1	12	*A.parasiticus*	+	8:A	45		_	13:E	78		_	19:E	111		_
2:B1	13	*A. flavus*	+	8:B1	46	*A. flavus*	+	14:A	79	*A. flavus*	+	20:A	112	*A. nidulans*	+
2:B2	14	*A. flavus*	+	8:D	47	*A.parasiticus*	+	14:B1+C	80	*A.parasiticus*	+	20:A	113	*A. flavus*	+
2:D	15		_	8:E	48		_	14:D	81		_	20:A	114	*A. flavus*	+
2:E	16	*A. flavus*	+	9:A	49	*A. flavus*	+	14:E	82		_	20:B2	115		_
2:E	17	*A. nidulans*	+	9:B2	50	*A. nidulans*	+	15:A	83	*A. flavus*	+	20:D	116	*A. flavus*	+
2:E	18	*A. nidulans*	+	9:B2	51	*A. flavus*	+	15:A	84		_	20:E	117	*A. nidulans*	+
3:A	19		_	9:D	52	*A. flavus*	+	15:B2	85	*A. nidulans*	+	21:A	118		_
3:B2	20	*A. flavus*	+	9:D	53	*A. flavus*	+	15:B2	86	*A. flavus*	+	21:B2	119	*A.parasiticus*	+
3:D	21		_	9:D	54		_	15:D	87	*A. flavus*	+	21:D	120		_
3:E	22	*A. flavus*	+	9:D	55	*A. flavus*	+	15:D	88	*A.parasiticus*	+	21:E	121		_
4:A	23	*A. flavus*	+	9:E	56	*A.parasiticus*	+	15:E	89		_	*F*	*ST*	*A. flavus*	+
4:A	24	*A. flavus*	+	10:A	57		_	16:A	90		_	*P*	*ST*	*A.parasiticus*	+
4:B2	25	*A.parasiticus*	+	10:A	58	*A. nomius*	+	16:B1	91		_				
4:D	26	*A. nidulans*	+	10:B1	59	*A. flavus*	+	16:B2	92		_				
4:D	27		_	10:B2	60		_	16:C	93		_				
4:E	28		_	10:B2	61	*A.parasiticus*	+	16:D	94		_				
5:A	29		_	10:D	62		_	16:E	95		_				
5:B2	30		_	10:E	63	*A. flavus*	+	17:A	96		_				
5:B2	31	*A.parasiticus*	+	11:A	64	*A. flavus*	+	17:B1	97	*A.parasiticus*	+				
5:B2	32	*A. flavus*	+	11:A	65		_	17:C	98		_				
5:D	33	*A. flavus*	+	11:B1+B2	66		_	17:D	99		_				

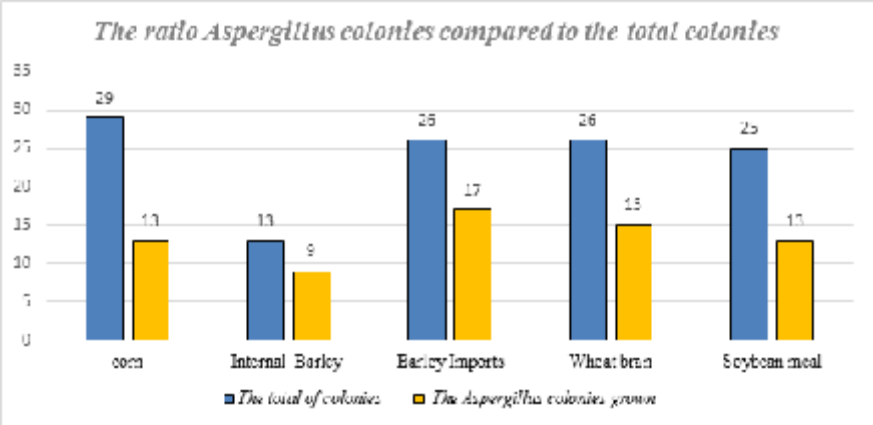

Fig. 4. The ratio of *Aspergillus* fungi colonies to the entire colonies

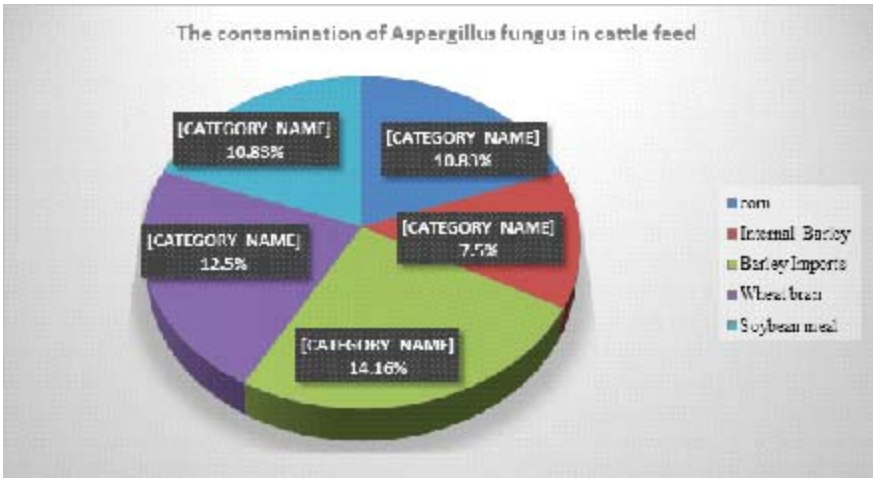

Fig. 5. The amount of *Aspergillus* fungi contamination in cattle feed

level of aflatoxin in cattle feed ration should be 20 ppb (STEP 2004). The surveys conducted in this study indicates that the highest contamination rates of cattle feed is belong to imported feeds which should not exceed this limit, generally this issue is important because consumption of contaminated cattle feed with *Aspergillus*, which exposed humans and animals to aflatoxins as a threat to the health of humans and animals (Smith *et al*. 1995; Kabak *et al*. 2006).

CONCLUSION

Since studies show that the most contamination of cattle feed is belong to imported cattle feed, so with the better management and supervision on the quality of imports, aerated silo and barn of cattle feed rations, training how to keep cattle feed rations to farmers and experts can be partially prevented pathogenic fungi toxins and prevent of Mycotoxin entry to animal and human health cycle, and finally we can sample in large scale and in different provinces until the results can be generalized to the whole country.

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