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DETERMINATION OF AFLATOXIN M_1 LEVELS IN BOVINE FARMED MILK WITH SPECIAL REFERENCE TO THE LEVELS OF AFLATOXIN B_1 IN THE ANIMAL FEED

(With 3 Tables)

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تحديد مستوى الأفلاتوكسين م1 فى ألبان المزارع البقرية مع الإشارة إلى مستوى الأفلاتو كسين ب1 فى أعلاف الحيوانات

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في دراسة لتحديد مستوى الأفلاتوكسين ب 1& م1 تم جمع 40 عينة من أربعة مزارع مختلفة من مدينة بورسعيد بواقع 20 عينة من كل من أعلاف الحيوانات وألبان الأبقار(5عينات ألبان و5 عينات أعلاف من كل مزرعة). ووجد ان متوسط مستوى الأفلاتوكسين ب 1 في عينات أعلاف الحيوانات كان 41,38 ± 9,6 ، 2,07 ±1,6 ، 7,48 ± 3,5 و1,19 ± 0,9 جزء في البليون، بينما كان متوسط مستوى الأفلاتوكسين م 1 في عينات الألبان البقرية 51,6 ± 10,8 ، 21,48 ±4,5 ، 35,72 ± 7,3 و8,63 ± 3,9 جزء في التريليون. وقد أسفرت النتائج عن وجود الأفلاتوكسين ب1 و الأفلاتوكسين م 1 في عينات أعلاف الحيوانات والألبان البقرية بنسبة 80% (16) ، 65% (13) علي التوالي. وبمقارنة مستوى الأفلاتوكسين ب 1& الأفلاتوكسين م1 في العينات موضع الدراسة وجد ان 25% (5) و20% (4) من كل من أعلاف الحيوانات والألبان البقرية علي التوالي قد تعدت الحد المسموح به طبقا لمنظمة FAO/WHO. وقد تم مقارنة مستوي الأفلاتوكسين ب 1 والأفلاتوكسين م 1 في العينات موضع الدراسة وكذلك مناقشة تأثير الأفلاتوكسين م 1 علي صحة المستهلك.

SUMMARY

A total of 40 samples of animal feed and milk (20 of each) were randomly collected from four small dairy farms and analyzed for aflatoxin B_1 and M_1 respectively. The mean average of AFB_1 and AFM_1 were 41.38 ± 9.6, 1.07 ± 0.6, 7.48 ± 3.5 and 1.19 ± 0.9 ppb; 51.6 ± 10.8, 21.48 ± 4.5, 35.72 ± 7.3 and 8.63 ± 3.9 ppt in the examined animal feed and milk samples respectively. The incidence of AFB_1 and AFM_1 was detected in 80% (16) and 65% (13) of the examined animal feed and milk samples respectively. On the other hand, 25% (5) and 20% (4) of the examined animal feed and milk samples were exceeded the limits recommended by FAO/WHO. The relationship between the levels of $AFB_1$and AFM_1in the examined animal feed and milk were discussed. Also the effect of AFM_1 on the human health was discussed. There is need to create awareness and establish routine monitoring of animal feeds and milk to reduce risk to animal and consequently human response.

Key words: *Aflatoxins, Aspergillus spp., milk, animal feed.*

INTRODUCTION

Aflatoxins (AFs) are secondary metabolites produced by some species of *Aspergilus*, especially *Aspergilus flavus* and *Aspergilus parasiticus*. They are naturally contaminants of food and several feedstuffs, initiated under favorable conditions of temperature, relative humidity/moisture and poor storage conditions with the long storage under favorable conditions molds produce their metabolites in the form of fungal toxins (IARC, 2002; Strosnider *et al.,* 2006).

Aflatoxins are a group of toxins classified into aflatoxin B_1, B_2, G_1 and G_2. These toxins are fluorescent under the ultraviolet light and don't have flavor and scent. They are resistant to high temperatures up to 320°C thus didn't exterminate or fragment by boiling and pasteurization (Early, 2000). The potential danger of aflatoxins group is the transfer of these toxins to human through food chain (Rory and Enda, 2008).

The toxicity of aflatoxin B_1 (AFB_1) in animals is varied due to differences in susceptibility (Lanza *et al.,* 1982). The toxic dose for cattle has been shown to be from 300–700 ppb depending up on the individual animal. The adverse effects include low weight gain and dietary intake causing growth impairment and depress the immune status (Raisuddin *et al.,* 1993).

In human, aflatoxin B_1 is the most toxic compound produced by some *Aspergilus* species and the most potent hepato-carcinogens. The International Agency for Research on Cancer of WHO cited that aflatoxin B_1 is carcinogenic of "Group 1" for humans (IARC, 2002). The long-term exposure to low levels of AFB_1 in the diet produced carcinogenic, mutagenic, teratogenic, oes-trogenic, neurotoxic and immunotoxic effect (Albert *et al.,* 2006).

In the liver of animal, AFB_1 was transformed biologically by hepatic microsomal cytochrome P450 into aflatoxin M_1 (AFM_1). Aflatoxin M_1 (AFM_1) is the principal hydroxylated AFB_1 metabolite present in milk of cows fed with a diet contaminated with AFB_1 (Battacone *et al.,* 2005) and excreted within 12 hours after administration of contaminated feeds (Battacone *et al.,* 2003; Applebaum *et al.,* 2003). The US Food and Drug Administration (FDA) have indicated that aflatoxin is the only mycotoxin that currently warrants regulation in milk (Wood and Trucksess, 1998).

Milk containing violative levels of AFM_1 cannot be marketed. The action level for most feeds is 20 ppb; the action level for human food is also 20 ppb for total aflatoxins, with the exception of milk which has an action level of 0.5 ppb for aflatoxin M_1. (FDA, 2005). the acute toxicity begins and seems to be similar or slightly less than that of aflatoxin B_1 but its carcinogenic potency is probably one or even two orders of magnitude lower than that of aflatoxin B_1 (Henry *et al.,* 2001).

This study was initiated to assess the levels of AFB_1 and AFM_1 in animal feeds and milk of different farms respectively, and the significant differences of parameters analyzed.

MATERIALS and METHODS

1- Samples collection:

1-1: Milk:

Milk samples (20) from four smallholder dairy farming households (5 of each) in Port-Said city were collected in sterile 15 milliliters tubes. All the samples were thoroughly identified and transported to the laboratory in cool boxes. The samples were analyzed as soon as possible and any delay, the samples should be frozen.

1-2: Animal feeds:

From the same smallholder dairy farming households, about 500g of animal feed were taken and thoroughly identified (20 samples) transported to the laboratory and analysed as soon as possible.

2- Sample preparation:

2-1: Milk:

Individual milk sample was centrifuged at 3500 rpm for 10 min. at 10°C for a process of de-fatting. By using a Pasteur pipette, the upper cream layer was removed completely, stored in cool place and protect against light (A.O.A.C., 2000).

2-2: Cereals and feed:

The cereal and feed sample was mixed and thoroughly ground. In a screw cap bottle 20 g of the mixed and ground sample was mixed with 10 ml of methanol: distilled water (70:30, v/v) by using shaker for 10 minutes at room temperature. The homogenate was filtered through Whatman® No. 1 filter paper. 100µ l of the sample filtrate was diluted with 600µ l of the sample dilution buffer. The diluted sample was stored in cool place and protect against light (Kang'ethe and Lang'a, 2009).

3- Enzyme immunoassay:

3-1: Aflatoxin M_1 (AFM_1) in milk:

In each well, 100 µl of the skimmed milk sample were taken and analyzed directly using an ELISA kit for M_1 following the manufacturer's instructions (Ridascreen® Aflatoxin M_1, purchased from r-biopharm, Germany). Samples were run in duplicates. The sample was diluted and re-tested when OD reading above the reading of 40 parts per trillion (ppt) standards. The kit had a sensitivity of 5ppt.

3-2: Aflatoxin B_1 (AFB_1) in cereals and feed:

50µl per well were employed directly in the assay. Competitive enzyme immunoassay for AFB_1 was done using Aflatoxin kit following the manufacturer's instructions (Ridascreen® Aflatoxin, Commercial License Eliza Kit, obtained from r-biopharm, Germany). Any sample having more than 13.5 ppb was diluted further (sample dilution buffer containing 10% methanol) and re-tested. This concentration was the upper limit of the sensitivity of the standard curve. The kit had a sensitivity limit of 1.8 ppb.

4- Calculation:

Special software, the RIDA®SOFT win (Art. No. Z9999) is available for evaluation of the RIDASCREEN® enzyme immunoassays.

The course of the slandered curve is shown in the quality assurance certificate enclosed in the test kit.
Remark for the calculation without software:

$$\frac{\text{Absorbance standard (or sample)}}{\text{Absorbance zero standard}} \times 100 = \%\ \text{absorbance}$$

5- Sensitivity:

The mean lower detection limit of the RIDASCREEN ® Aflatoxin total assay is about 50 ng/kg (0.5 ppb) for milk. According to the test preparation record, the detection limit is (1.75ppb) for cereals and feed.

6- Statistical methods:

Data were submitted to one-way ANOVA to test differences between treatments, and linear regression analysis was carried out to test AFM_1 and AFB_1 (SPSS, 2007).

RESULTS

Table 1: Quantitative estimation of aflatoxins $B_1$and M_1 in the examined animal feed and milk samples in different farms (n=5).

No. of sample	Farm 1		Farm 2		Farm 3		Farm 4	
	$AFLB_1$ (ppb)	$AFLM_1$ (ppt)	$AFLB_1$ (ppb)	$AFLM_1$ (ppt)	$AFLB_1$ (ppb)	$AFLM_1$ (ppt)	$AFLB_1$ (ppb)	$AFLM_1$ (ppt)
1	26.43	53.01	3.55	41.12	1.05	27.76	1.794	22.04
2	12.29	54.61	1.750	23.35	1.259	30.46	0.038	15.31
3	45.20	58.03	N/D	19.19	12.048	42.24	N/D	N/D
4	90.20	58.08	0.039	23.74	20.770	44.61	0.116	5.78
5	32.8	34.37	N/D	N/D	2.276	33.53	N/D	N/D

ppb = part per billion
ppt= part per trillion
N/D=not detectable

Table 2: Statistical analytical results of aflatoxins B_1 and M_1 in the examined animal feed and milk samples in different farms (n=5).

Farms	Statics	$AFLB_1$ (ppb)	$AFLM_1$ (ppt)
Farm1	Min.	12.29	34.37
	Max.	90.20	58.08
	Mean ± SE	41.38 ± 9.6	51.6 ± 10.8
Farm2	Min.	0.039	19.19
	Max.	3.55	41.12
	Mean ± SE	1.07 ± 0.6	21.48 ± 4.5
Farm3	Min.	1.05	27.76
	Max.	20.770	44.61
	Mean ± SE	7.48 ± 3.5	35.72 ± 7.3
Farm4	Min.	0.038	5.78
	Max.	1.794	22.04
	Mean ± SE	1.19 ± 0.9	8.63 ± 3.9

Table 3: Incidence of aflatoxin B_1 and M_1 in the examined animal feeds and milk samples in different farms. (n=5).

Farm No.	$AFLB_1$in animal feed						$AFLM_1$in milk					
	Contaminated samples		Samples exceed PMls		Samples (ND)		Contaminated samples		Samples exceed PMls		Samples (ND)	
	+ve	%	+ve	%	+ve	%	+ve	%	+ve	%	+ve	%
1	5	100	4	80	0	0	5	100	4	80	0	0
2	3	60	0	0	2	40	1	20	0	0	1	20
3	5	100	1	20	0	0	5	100	0	0	0	0
4	3	60	0	0	2	40	2	40	0	0	2	40
Total	16	80	5	25	4	20	13	65	4	20	3	15

N/D =not detectable
PMls =Permissible limits

DISCUSSION

A total of 20 animal feeds samples were collected from 4 farms (5 of each). The results tabulated in Table 1 showed that the highest recorded levels of aflatoxin B_1 and M_1 were 90.20 ppb and 58.08 ppt in the examined feed animal and milk samples of farm 1 respectively, while the lowest levels were 0.038 ppb and 5.78 ppt in feed animal and milk samples of farm 4 respectively. The results of feed animal samples were agree with the results recorded by Karki and Sinha, (1989) but not agreed with the result of Abdel-Fattah and Yacoub, (2008) who stated that aflatoxin not detected in animal feed. The variation in the levels of aflatoxin B_1 between the different samples and farms may be attributed to the differences in storages facilities of animals' feeds, temperature, humidity and high insect activity (Strosnider *et al.,* 2006). On the other hand, milk samples results were agree with the results of Battacone *et al.* (2003) but disagree with the results recorded by Westlake *et al.* (1989). Also, Table 1 showed a significant relationship between the levels of aflatoxin B_1 and M_1 in animal feed and milk samples respectively. Thus Aflatoxin M_1used as possible marker of exposure to aflatoxin B_1, as the amount of AFB_1 ingested increased the AFM_1 concentration in milk rose. These results agree with the result of Veldmann *et al.* (1992); Michael *et al.* (2002) but not agree with that of Frobish *et al.* (1986). The significant relationship between the levels of aflatoxin B_1 and M_1 may be regards to that the considerable part of the ingested aflatoxin B_1 is degraded in the rumen and extensively metabolized in the liver, resulting predominantly in aflatoxin M_1. Circulating aflatoxin M_1 can be excreted via the kidneys and appears in milk. The excreted amount of aflatoxin M_1 in the milk of dairy cows was estimated to represent 1-2 % of the ingested aflatoxin B_1. Also the changes in the plasma- milk barrier result in a higher carry over rate of aflatoxin M_1 into milk (Munksgaard *et al.,* 1987; Veldmann *et al.,* 1992).

As shown in Table 2 the mean levels of aflatoxin B_1 and M_1 in the examined animal feed and milk samples were 41.38 ± 9.6, 1.07 ± 0.6, 7.48 ± 3.5 and 1.19 ± 0.9 ppb; 51.6 ± 10.8, 21.48 ± 4.5, 35.72 ± 7.3 and 8.63 ± 3.9 ppt, respectively in the four farms. The variation between the levels of aflatoxin B_1 in different farms and within the same farm is regarded to the variation of the feeds nutrient content of the different farms i.e. feed manufactured from grains is considered the major source for aflatoxin B_1 contamination that find their way to animal feed. Also

high temperature, humidity and moisture content induce the mold growth and aflatoxin B_1 production in animal feed (IARC, 2002).

The obtained results recorded in Table 3 showed that 80% (16) and 65% (13) of the examined animal feeds and milk samples were contaminated with aflatoxin B_1 and M_1 respectively. This result is nearly agreed with the results recorded by Rory and Enda (2008), they reported that eighty six percent of the feed samples from farmers were contaminated with aflatoxin B_1. Contamination of animal feeds with AFB_1 was prevalent throughout the farms. 20% (4) of samples were free from AFB_1, Meanwhile, 25% (5) of the contaminated samples had aflatoxin B_1levels above FAO/WHO limits **(**20 ppb), and also 20% (4) of contaminated milk samples had aflatoxin M_1 levels above FAO/WHO limits0.05µg/Kg (50 ppt). (FAO/WHO, 2002). These results confirm unavoidable contaminants in food and feed, so AFB_1 level must be lowered 20 ppb of total aflatoxin in animal feed to avoid its harmful effects for the animals or human who consume its products. Total rations containing aflatoxin at levels greater than 20 pbb certainly should not be fed to milk cows. In this concern, the Food and Agriculture Organization (1996) reported that 25% of the world's food crops are affected by mycotoxins.

CONCLUSION

These results suggest that presently the contamination of milk with AFM_1 does not appear to be a serious health problem in Port-said, and may be a direct and immediate correlation between the presence of AFB_1 in feedstuff and the detection of AFM_1 in milk from the same farm. It is necessary to highlight the procedures in place for minimizing potential risks to animals and subsequent risks to consumers from animal derived food products. Aflatoxin M_1 contamination of milk cannot be completely prevented because AFB_1 does occur naturally in grains. It is not practical to completely eliminate AFB_1 from feeds or AFM_1 from milk. However, it is possible to control the amount of AFM_1 present in milk by limiting the amount of aflatoxin in animal feeds. The FDA has established specific guidelines on acceptable levels of aflatoxins in human food and animal feed by establishing action levels that allow for the removal of violative lots from commerce (Smith, 2005). Nevertheless, a continuous surveillance programme may be warranted to monitor regularly the occurrence of aflatoxin in the animal feeds

responsible for current limited contamination and to note rapidly any worsening in the situation that may depend on market changes or on unfavorable climatic developments

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