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Determination of Aflatoxins and Ochratoxin a in Dairy Cattle Feed and Milk in Wad Medani, Sudan

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Abstract: Aflatoxins and Ochratoxins are highly carcinogenic agents consistently found as food and feed contaminants leading to health risks in both human and animal. The aim of this study was to determine prevalence of aflatoxins and ochratoxin A contamination and their levels in dairy cattle feed and milk samples in Wad Medani, central Sudan. Validated and specific methods were used for extraction of aflatoxins and ochratoxin A from both feed and milk, then separated and determined by High Performance Liquid Chromatography (HPLC). The samples were collected from five farms in al Bohoth, al Kiraiba, Atrah, Hantoub and Brakat according to type of feeding and examined. The results showed that 6 out of 9 (66.67%) of the feed samples were contaminated by aflatoxins at concentration ranging between 2.79 and 147.13 μg kg^{-1}, while ochratoxin A was detected in 7 out of 9 (77.78%) of the samples concentrations ranging from 0.22-1.59 μg kg^{-1}. Aflatoxin B1 (AfB1) was the most common contaminant which was detected in 5 out of 9 (55.56%) samples followed by Aflatoxin G1 (AfG1) and Aflatoxin G2 (AfG2) both were detected in 2 out of 9 (22.22%) samples and aflatoxin B2(AfB2) which was detected in 1 out of 9 (11.11%) samples. Aflatoxin M1 (AfM1) was detected in 3 out of 5 (60.00%) milk samples at average concentration of 0.16 μg L^{-1}, whereas ochratoxin A was detected in 1 out of 5 (20.00%) (2.73 μg L^{-1}) milk samples.

Key words: Aflatoxin, ochratoxin A, dairy cattle feed, milk, HPLC, Medani, Sudan

INTRODUCTION

Aflatoxins are toxic, mutagenic and carcinogenic compounds which contaminate various types of food and feedstuff (Murphy *et al*., 2006) and produced by certain strains of *Aspergillus flavus* and *Aspergillus parasiticus* (Wilson and King, 1995; Whitlow and Hagler, 2002). Ochratoxin A is produced by some species of fungi including *Aspergillus ochraceus*, *Aspergillus niger* and *Penicillium verrucosum* (Batista *et al*., 2009), It is nephrotoxic and hepatotoxic and is likely to have carcinogenic potential in humans (Shundo *et al*., 2009a).

Animal exposure to mycotoxins causes a variety of symptoms depending on the animal species. However, in all animals, aflatoxins can cause liver damage, decreased reproductive performance, reduced milk or egg production, embryonic death, teratogenicity, tumors and suppressed immune system function, even when low levels are consumed (Akande *et al*., 2006). AfB_1 has been shown through research to be the most potent naturally occurring carcinogen in animals, with a very strong link to human cancer incidence (Verma, 2004; Santacroce *et al*., 2008).

Aflatoxin M1 (AFM1), the major metabolite of Aflatoxin B1 (AFB1) and Ochratoxin A (OTA) are classified by the International Agency of Research on Cancer as class 2B, possible human carcinogens (Boudra *et al*., 2007).

The USA Food and Drug Administration (FDA) has established 20 μg kg^{-1} and 0.5 μg L^{-1} as action levels for aflatoxin present in animal feed and milk respectively (Khanafari *et al*., 2007). Wad Medani is the largest city in central Sudan with substantial population of dairy cattle. The present investigation in this area aimed to determine the situation and levels of aflatoxins and ochratoxin A in dairy cattle feed and milk.

MATERIALS AND METHODS

Nine feed samples (1-2 kg of weight) and five of fluid bulk milk (1000 mL of volume) were collected from five dairy farms in al Bohoth, al Kiraiba, Atrah, Hantoub and Brakat according during March, 2009. The samples were kept at -20°C deep-freezer till tested. At the time of analysis samples were brought up to room temperature (Richard *et al*., 1993).

Extraction of aflatoxins from feed: Aflatoxins were extracted and determined as described by Elzupir *et al*. (2009) using HPLC adopting UV Detection at 365 nm.

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Extraction of aflatoxin M1 from milk: Aflatoxin M1 was extracted using AOAC official method 980.21. with some minor modification. In brief; 30 mL chloroform and 5 mL salt solution (10 g NaCl into 50 mL H_2O) was added into 50 mL falcon tube containing 10 mL of milk, securely stoppered, shaked gently and centrifuged at 1500 rpm for ten minutes. The volume of chloroform extract was recorded, then cleaned up by passing it through glass column (10×250 mm) packed with silica gel (2 g) in chloroform solvent using Na_2SO_4 (0.5 g) to cap column. The column was washed with 7 mL toluene: acetic acid (9: 1) and 7 mL hexane: diethyl ether: acetonitrile (5: 3: 2). Aflatoxin M1 was then eluted with 10 mL chloroform: acetone (4: 1) and evaporated to near dryness; carefully transferred into screw capped borosilicate vial and evaporated to dryness. The dry film was then dissolved with 500 μL mobile phase solution and injected into HPLC. The chromatographic conditions (Jonsyn *et al*., 1995) were as follows:

- Column type and size: C18; 250 X 4.6 mm I.D.; 5 micron particle size
- Temperature: ambient temperature 25°C
- Fluorescence detector: 365 and 418 nm as wavelengths for excitation and emission, respectively
- Mobile phase: Methanol: water: acetic acid (65: 35: 1)
- Flow rate: 1 mL min^{-1}
- Injection Volume: 20 μL

Calibration curve was determined, using series of dilutions containing 600, 1200 and 2400 pg mL^{-1} of aflatoxin M1 standard. The correlation factor was 0.981.

Extraction of ochratoxin A from feed: Extraction, clean up and determination of ochratoxin A was done using AOAC official method 991.44. In brief; feed powder (50 g) was transfered into 500 mL conical flask and 250 mL chloroform, 25 mL 100 mM phosphoric acid were then added. The flask was securely stoppered and shaken on a wrist action shaker for 30 min and filtered through filter paper. About 50 mL of the filtrate was then transferred into a separation funnel and extracted with 10 mL 3% sodium bicarbonate and the upper (bicarbonate) phase was collected. About 5 mL bicarbonate extract were then loaded into C18 cartridge that has been previously washed two times with 2 mL methanol, 2 mL water and 2 mL 3% sodium bicarbonate. C18 column was then washed with 2 mL phosphoric acid and 2 mL water and ochratoxin A was eluted with 8 mL ethyl acetate: methanol: acetic acid (95: 5: 0.5) into screw capped borosilicate vial containing 2 mL water. The vial was shaken until the two phases were completely mixed and left for approximately 2 min. The upper phase was then collected into new screw capped borosilicate vial. The lower phase was washed twice with 1 mL ethyl acetate to extract ochratoxin A left in a fraction of upper phase remaining in the lower phase, both fractions were combined to upper phase and then evaporated to dryness. The dry film was dissolved with 500 μL mobile phase and injected into HPLC. The chromatographic conditions were as follows:

- Column type and size: C18; 250 X 4.6 mm I.D.; 5 micron particle size
- Temperature: ambient temperature 25°C
- Fluorescence detector: 333 and 460 nm as wavelengths for excitation and emission, respectively
- Mobile phase: acetonitrile:water:acetic acid (99: 99: 2)
- Flow rate: 1 mL min^{-1}
- Injection Volume: 20 μL

Calibration curve was determined, using series of dilutions containing 2, 4 and 8 ng 20 μL^{-1} of ochratoxin A standard. The correlation factor was 0.999.

Extraction of ochratoxin A from milk: Ochratoxin A was extracted and determined as described by Gonza_lez-Osnaya *et al*. (2008). In brief; 5 mL methanol were added to 15 mL falcon tube containing 5 mL milk sample. The tube was securely stoppered, shaken gently and centrifuged at 3000 rpm for 40 min, filtrated and evaporated to dryness. The dry film was then redissolved in 500 μL mobile phase and injected into HPLC. The chromatographic conditions were the same as those described above for ochratoxin A in feed.

RESULTS AND DISCUSSION

The results were shown in Table 1 and 2. Aflatoxin were detected in six feed samples; five of which contained aflatoxin level that were higher than the accepted level of 20 μg kg^{-1} in animal feed (Khanafari *et al*., 2007).

Manufactured ration samples; which forms the main ration in farm A; showed contamination level of 76.16 μg kg^{-1}. This level of contamination was alarmingly high, however the disparity in aflatoxin contamination levels between farm A (76.16 μg kg^{-1}) and farm B (2.79 μg kg^{-1}) may reflect that the rations were stored for longer periods in farm A.

The oily feeds (Groundnut cake meal and Sunflower cake meal), which are used in farms C, D and E as raw ingredients showed high aflatoxins contamination levels that ranged between 70.12 and 147.13 μg kg^{-1} (Table 1). The oily rations substrates are known to be more

Table 1: Prevalence of different aflatoxin types and ochratoxin A in dairy cattle feed

Farms ID	Sample ID	Sample type	AfB1 (μg kg^{-1} ±0.05)	AfB2(μg kg^{-1} ±0.06)	AfG1(μg kg^{-1} ±0.08)	AfG2 (μg kg^{-1} ±0.01)	Aflatoxin total	Ochratoxin A (μg kg^{-1})
A	8	Manufactured Ration	76.16	-	-	-	76.16	0.22
B	16	Manufactured Ration	2.79	-	-	-	2.79	0.61
C	2	Crushes Sorghum	-	-	-	-	-	1.58
	1	Sunflower cake meal	19.20	-	-	61.79	80.99	1.59
D	14	Crushes Sorghum	21.88	0.08	-	-	21.97	0.33
	13	Wheat bran	-	-	-	-	-	0.43
	12	Groundnut cake meal	74.96	13.05	59.12	-	147.13	0.31
E	18	Groundnut cake meal	-	-	70.12	-	70.12	-
	19	Wheat bran	-	-	-	-	-	-

-: Not detected

Table 2: Prevalence of aflatoxin M1 and ochratoxin A dairy cattle milk

Farm ID (μg L^{-1})	A	B	C	D	E
Aflatoxin M1	0.11	-	-	0.25	0.11
Ochratoxin A	-	2.73	-	-	-

-: Not detected

susceptible to fungi growth and aflatoxin production (Wild *et al*., 1993). On the other hand, crushed sorghum showed the least value of contamination (21.97 μg kg^{-1}) whereas wheat bran was free from aflatoxin. Furthermore, it could be shown from Table 1 that AfB1 was the most common contaminant being detected in 5 out of 9 (55.56%) samples, followed by AfG1 and aflatoxin AfB2 both were detected in 2 out of 9 (22.22%), respectively and lastly aflatoxin AfG2 which was detected in 1 out of 9 (11.11%) samples. The results disclosed in the present investigation are consistent with that obtained in Khartoum state (Elzupir *et al*., 2009) but higher than those estimated by Abdel-Rahim *et al*. (1989), Younis and Kamal (2003) and Elamin *et al*. (1988) in various food and feed stuffs in Sudan. The different values obtained in different studies may be due to differences in the techniques used and types of samples analyzed.

AfM1 was detected in 3 out of 5 (60%) bulk milk samples with an average concentration of 0.16 μg L^{-1}, this value is lower than the accepted level (0.5 μg L^{-1}) established by Brazilian Ministry of Health (Shundo *et al*., 2009b) but higher than the level (0.05 μg L^{-1}) established by the European Union that is considered one of the lowest in the world (Boudra *et al*., 2007). The presence of AfM1, the major metabolite of AfB1, in milk samples paralleled the high levels of AfB1 contamination noticed in farms A and D. In this respect farm E was an exception in that no AfB1 was detected in the sample obtained from this farm. This might indicate that other ingredients (not tested in the present study) offered to animals in this farm might have been contaminated. On the other hand, farms B and C showed low level of AfB1 contamination, consequently the levels of AfM1 excreted in the milk samples from these farms might have been too low to be detected by the method.

Ochratoxin A was detected in 7 out of 9 (77.78%) feed samples with an average concentration of 0.72 μg kg^{-1} and in 1 out of 5 (20%) milk samples with a concentration of 2.73 μg L^{-1}. These contamination levels by ochratoxin A are alarmingly high in view of the fact that the Tolerable Daily Intake (TDI) set by the European Union (EU), the Scientific Committee for Food (SCF) is 5 ng kg^{-1} bw/day (Gumus *et al*., 2004). In contrast, no regulation for OTA in milk exists (Boudra *et al*., 2007).

CONCLUSION

Finally, in agreement with the previous observations in Khartoum state (Elzupir *et al*., 2009), the present investigation showed the high prevalence of aflatoxin contamination in animal feed in Medani-Central Sudan and to the knowledge, the present study represents the first record on Ochratoxin contamination in Sudan.

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