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Full Length Research Paper

Co-occurring mycotoxins in animal feeds

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Mycotoxin contamination of feeds results in economic loss and transmission of toxins in the food chain. Animal feeds, the raw ingredients used in their manufacture, namely, maize, wheat, sunflower seeds, cottonseeds, bagasse, wheaten bran, gluten feed and pet foods from South Africa were surveyed for contaminating mycotoxin-producing fungi and their toxins: aflatoxins, fumonisins, zearalenone and ochratoxins. Toxins were extracted and analysed by high performance liquid chromatography and fluorometry. Twenty-one of the twenty-three samples were contaminated by *Aspergillus flavus* which co-occurred with *A. parasiticus* in two samples, *A. tamarii* in seven and *Fusarium moniliforme* in one. *Rhizopus stolonifer, R. oryzae* and yeast were also isolated. Aflatoxins were detected in seventeen samples, fumonisin in six and zearalenone in three. Aflatoxin levels ranged between 0.8 ± 0.2 and 156 ± 8 µg/kg (ppb), zearalenone between 100 ± 10.5 and 165 000 ± 200 µg/kg and fumonisin B_1 between 15 ± 3.0 and 5 900 ± 40 µg/kg. Ochratoxins were not detected in any of the samples. In most countries worldwide, legislated levels for aflatoxins and patulin are 20 µg/kg and 50 µg/kg, respectively, for human foods. Fumonisins, zearalenone and other toxins are not legislated in most of the countries. Ten of the feeds contain toxin levels above legislated limits (for Canada and the USA) and guidelines set by other countries. The results of this study highlight the need for mycotoxin legislation in the animal feed industry.

Key words: Animal feed, pet foods, aflatoxins, fumonisins, zearalenone, ochratoxin

INTRODUCTION

Animal feeds are an essential part of the farm animal to human food chain; therefore, infectious and non-infectious hazards present in animal feeds pose a threat to human health. Public concern on health matters related to food has increased following the recent epidemic of bovine spongiform encephalopathy (BSE) in the United Kingdom and foot and mouth disease in United Kingdom, Ireland, Belgium, France, Germany and South Africa (Hinton, 2000). Animal feeds provide a market for slaughterhouse offals, grains considered “unfit for human consumption” and similar waste products to be turned into a profit. Cereal grains are the primary ingredients of most animal feeds and these are often of sub-standard grade, which predisposes these grains to mycotoxin contamination. Mycotoxins are a diverse group of low molecular weight metabolites produced by naturally-occurring fungi and are harmful to man and animals (Meronuck and Xie, 1999).

Mould infection and mycotoxin contamination of cereal grains can occur in the field during growing, at harvest and during storage. As the grains progress through harvesting and storage, feed manufacture and delivery to farms, the level of mycotoxin contamination generally increases. The severity of mycotoxin contamination varies considerably. It is promoted by excessive moisture, temperature extremes, humidity, drought, variation in harvesting practices and insect damage (Gotlieb, 1997). Although over 300 mycotoxins have been identified, the toxicology of a few have been established for several animal species. The signs of the many mycotoxicoses are diverse, numerous and often dependent on species, sex, age, stress, reproductive and health status of the animal. This aspect has been reviewed extensively (Krogh, 1989; Miller, 1991; 1993; Wogan, 1991; Battalgia et al., 1996) and the symptoms include: feed refusal and vomiting (deoxynivalenol), impaired reproductive function and reduced fertility (zearalenone, deoxynivalenol, T-2 toxin), nephrotoxicosis (ochratoxin A and

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fumonisins), neurotoxicosis (fumonisins, cyclopiazonic acid), lung disease (fumonisins), hepatotoxicosis (fumoni-sins, cyclopiazonic acid), cancer (aflatoxins, T-2 toxin and fumonisins) and death (aflatoxins, T-2 toxin, fumonisins and ochratoxin A). Other effects of mycotoxin contamination are reduced immune function with compromised resistance to infection and disease (deoxynivalenol, aflatoxins and ochratoxin A) and reduced animal performance (deoxynivalenol, aflatoxins, T-2 toxin and ochratoxin A).

The severity of the response to mycotoxins depends to a large extent on the specific mycotoxin present and the level of contamination. Studies have shown that various mycotoxins present in combination have more severe effects than individual mycotoxins alone (Friend et al., 1992; Harvey et al., 1996; Kubena et al., 1997a; b). There is little information available regarding the prevalence and concentration of mycotoxins in foods of animal origin, although some toxins ingested by animals may be found in meat, milk or eggs (Park and Laing, 1993; Dorner et al., 1994). The worldwide problem of mycotoxicosis (Oldenburg, 1991; Jelinek et al., 1998; Scudamore and Livesey, 1998) is reflected by the fact that over 60 countries have either legislation or proposed legislation for the control of myco-toxins in both animal feeds and human foods (Vanegmond, 1995). However, there is no consistent rationale for setting limits or for enforcement of control measures. In this study, raw ingredients for animal feed manufacture, feeds and pet foods were surveyed for contaminating fungi and mycotoxins in order to assess the severity of mycotoxin contamination in South Africa. Analyses of this type will serve to highlight the urgency for the establishment of guidelines and legislated limits for all toxins in animal feeds.

MATERIALS AND METHOD

Samples

Twenty-three feed samples from KwaZulu Natal province of South Africa were analyzed. Six brands of agricultural feeds (broiler finisher pellets, dairy 15 pellets, layer 115 mash , layer 16 mash, mixed fowl feed and poultry growing mash) , eleven different raw ingredients that are used in animal feed production (yellow whole maize, sifted crushed maize, sunflower seeds, cotton seed oil cake, yellow powdery maize, sorghum brewers grains, gluten feed, wheaten bran, sunflower oil cake, molasses meal and bagasse) and six brands of commercially available pet foods (three pelleted pet foods and three canned pet foods) were used in this study. Sub-samples of each of the twenty-three samples were extracted and analyzed in triplicate.

Isolation of fungi

Aliquots of 0.5 g of each sample were placed on chloramphenicol agar (Biolab, Midrand, SA) and incubated at 25°C for 7 to 12 days. The prevalent moulds that developed were enumerated and sub-cultured on Sabouraud dextrose agar (Oxoid, New Hampshire, USA) for isolation of pure cultures. Pure cultures were identified using the light and the dissecting microscope and fungal identification keys for *Aspergillus* spp. (Klich and Pitt, 1988), *Fusarium* spp. (Nelson et al., 1983) and other common fungi (Beneke and Stevenson, 1987).

Toxin analysis

A Series-4 fluorometer (BBI-Source Scientific Inc., California, USA) was used to quantify aflatoxins, zearalenone and ocratoxin A. The toxins were separately extracted and purified by immunoaffinity chromatography as described in the Vicam Instruction Manuals for Aflatest, Zearalatest and Ochratest (Vicam, Watertown, USA). The efficiency of the extraction protocols was determined by spiking the pellet pet food, bagasse and sunflower oil cake sample with 40 µg/g of each of the toxin standards (zearalenone, ochratoxin A and aflatoxins), extracting and purifying the toxins and comparing toxin levels.

Fumonisins were extracted and purified separately using the method of Thiel et al. (1993). Extraction efficiency for fumonisin B_1 was also determined for the three samples described above. Fumonisin B_I was analysed by high-performance liquid chromatography (HPLC) using a Merck-Hitachi Model 7000 HPLC system equipped with a L-7400 variable wavelength UV detector (Merck-Hitachi) set at 276 nm, a L-7200 auto sampler (Merck-Hitachi) and a L-7100 pump (Merck-Hitachi). The mobile phase was methanol: sodium dihydrogen phosphate (68:32 v/v).

Detection limits for aflatoxins, zearalenone, and ochratoxin were determined with the fluorometer and that of fumonisins by HPLC and were respectively 4 µg/kg for zearalenone, 6 µg/kg for ochratoxin A, 4 µg/kg aflatoxins and 5 µg/kg for fumonisin B_1. All the analysis were carried out in triplicate and the results were expressed the mean ±SD.

RESULTS AND DISCUSSION

All the sample analysed were contaminated by fungi. Of the 23 samples, 21 were contaminated by the mycotoxigenic fungus, *Aspergillus flavus* and the other two by *Rhizopus oryzae*. Other mycotoxigenic fungi that were co-occurring with *A. flavus* were *A. parasiticus* in two samples, *A. tamarii* in seven samples and *Fusarium moniliforme* in one sample (Table 1). The predominance of *A. flavus* could be due to physiological characteristics, which enable it to survive adverse conditions. It is also a rapidly growing (Onions et al., 1981), temperature-tolerant fungus (Davis and Diener, 1983) that can withstand low moisture levels (Meronuck and Xie, 1999). *A. flavus* was also detected in two of the three canned pet foods tested, which was unexpected as canned foods have a high acidity and contain food preservatives. *A. parasiticus* was only detected in yellow whole maize and the pellet pet food sample. This result is in accordance with the growth characteristics of this fungus. *A. parasiticus* is not as ubiquitous nor does it grow as rapidly as *A. flavus* (Meronuck and Xie, 1999). Another important point to consider is that it is more commonly found as a contaminant in peanuts and none of the 23 samples analysed contained any peanuts. *A. tamarii* was detected in seven of 23 samples. It is, therefore, not as prevalent as *A. flavus*, as corroborated by other studies (Onions et al., 1981). The fumonisin-producing fungus, *F. moniliforme*, was only found in one sample, yellow whole maize. *F. moniliforme* is a slow growing fungus and was out-competed by *A. flavus*. Two other fungal species, both non-toxigenic, were identified, namely, *Rhizopus stolonifer* and *R. oryzae*. The raw ingredients were more heavily contaminated with a greater number of fungal species than processed food and feed samples. This is not surprising since some of the fungal species that are not as resistant to thermal treatment and

Table 1. Incidence of fungi and toxin levels in feeds and pet foods.

Animal Feed	Fungal growth in mm (a, b)						Mycotoxin levels in µg/kg of Animal Feed (ppb) (a,b)		
	A. flavus	*A. parasiticus*	*A. tamarii*	*F. moniliforme*	*R. stolonifer*	*R. oryzae*	Aflatoxins	Fumonisin B_1	Zearalenone
Yellow whole maize	22	13	-	12	-	-	14.8±1.6	5 900±4	-
Sifted crushed maize	40	-	-	-	32	-	11.2±1.8	15±0.3	-
Yellow powdery maize	39	-	-	-	34	-	60±0.6	497±7	2 500±30
Sunflower seeds	19	-	-	-	-	33	5.6±0.7	-	-
Cotton seed oil cake	23	-	-	-	-	35	80±0.4	-	-
Sorghum brewers grains	25	-	22	-	-	-	0.8±0.2	-	-
Gluten feed	21	-	32	-	-	-	13.2±1.6	-	-
Wheaten bran	22	-	-	-	-	35	12±0.5	-	100±1.5
Sunflower oil cake	32	-	12	-	13	31	84±2.6	-	-
Molasses meal	31	-	11	-	-	21	84±5	-	-
Bagasse	20	-	22	-	-	23	156±0.8	-	165 000±200
Broiler finisher pellets	31	-	12	-	-	-	10.7±0.4	226±6	-
Dairy 15 pellets	20	-	-	-	-	35	64±2.1	-	-
Layer 115 mash	33	-	-	-	22	-	38±0.4	-	-
Layer 16 mash	33	-	-	-	-	23	12.4±0.5	-	-
Mixed fowl feeds	31	-	-	-	-	-	13.4±1.4	20.5±1	-
Poultry growing mash	23	-	-	-	33	-	14.7±4	474±4	-
Pelleted Dog Food (brand A)	11	12	-	-	21	-	-	-	-
Canned Dog Food (brand B)	11	-		-	-	-	-	-	-
Pelleted Cat Food (brand C)	13	-	12	-	-	23	-	-	-
Canned Cat Food (brand D)	12	-		-	-	-	-	-	-
Pelleted Dog Food (brand E)		-		-	-	22	-	-	-
Canned Dog Food (brand F)		-		-		22	-	-	-

a: Mean values obtained from experiments performed in triplicate.
b: Mean value determined graphically and standard deviation.

drying would have been eliminated during processing.

Toxin analyses were validated with spiked samples. The average recovery rates (n = 2) for the pellet, bagasse and sunflower oil cake samples, respectively were: aflatoxins 90, 87 and 79%; zearalenone was 81, 77 and 75%, ochratoxin was 89, 87 and 78% and fumonisin B_1 was 91, 89 and 85%. Almost all the feed samples were contaminated by fungi but the correlation between the fungi and their respective toxin/s was not always present. *A. flavus* was the predominant fungus isolated, and this fungus is known to produce aflatoxin B_1 and B_2. However, in this study preliminary screening by thin layer chromatography (results not shown) revealed that aflatoxin B_2 was present in 14 samples, G_1 in one and G_2 in five samples. B_1 was not detected in any of the samples. Bhatnagar et al. (1991) showed

that aflatoxin B_1 and B_2 are synthesized in fungal mycelia by separate pathways, indicating a branch point in the pathway. The predominance of aflatoxin B_2 could be due to mutations, which have been previously reported (Mirocha et al., 1979). Despite the presence of *A. flavus* in the pet food samples, no toxins were detected. The low moisture content, low pH and the presence of preservatives may have suppressed the production of these toxins. Aflatoxins are known to be susceptible/degraded by heat (Rehana and Basappa, 1990) and physical and chemical methods (Samarajeewa et al., 1990), therefore, if present initially may have been eliminated during processing and canning.

Aflatoxin levels ranged from 0.8 ± 0.2 to 156 ±0. 8 µg/kg. Seven of the 23 samples had levels equal to or above legislated limits, which is 20 µg/kg for animal feeds in Canada and the USA. Cotton oil seed cake, sunflower oil cake and molasses meal had at least four times this level while bagasse was even more heavily contaminated being almost eight times over the legislated limits for Canada and the USA. In South Africa only two toxins, aflatoxins and patulin have legislated limits for food for human consumption (Sibanda et al., 1997). In animal feeds, there merely are guidelines for these and other toxins levels.

Ochratoxin A was not detected in any of the agricultural feeds or their raw ingredients. Zearalenone was detected in three of the raw ingredients (yellow powdery maize, wheaten bran, and bagasse) in levels ranging from 100 ± 1.5 to 165 000 ± 200 µg/kg although the producing fungus, *F. graminearum*, was not isolated from these feeds. Zearalenone is regarded as the most troublesome and prevalent *Fusarium* mycotoxin, which seriously reduces the quality of cereals and grain products (FAO et al., 1999). Sixteen countries (mostly European) have set guidelines for zearalenone levels in cereals ranging from 50 to 1000 µg/kg (Zinedine et al., 2007). However, this toxin is not regulated in many countries including South Africa. In this study zearalenone levels as high as 165 000 µg/kg were detected in bagasse. This is over 16.5 times the level regarded as safe for animal consumption. The highest aflatoxin levels were also found in bagasse.

The fumonisin-producing fungus *F. moniliforme* (*verticillioides*) was only found in whole maize sample, but fumonisin B_1 was found in several raw ingredients and feed samples (Table 1). The highest fumonisin B_1 level (5 900 µg/kg) was detected in yellow maize and is above the maximum level in maize (5 000 µg/kg) established by the FDA for small animals. Even processed pellets had high levels of fumonisin B_1. This toxin is very stable even when treated by high temperatures and chemicals (Jackson and Bullerman, 1999). A recent worldwide survey for fumonisins in corn and corn-based products for human consumption found on average > 50% incidence of this toxin, but 77% for Africa and 53% for Europe. Over 82% of animal feeds in the USA and Europe were contaminated by fumonisins and 72% of the samples at > 1 mg/kg (Visconti, 2001). Given the higher incidence of this toxin in foods in African countries, the probability of higher incidences in animal feeds is also high.

Rapid and sensitive tests kits for mycotoxins are available to farmers and consumers. However, without guidelines, control and regulation is difficult. The results show that almost all feed and pet foods have fungal contaminants as well as several toxins. Seventeen of the 23 samples tested positive for toxins and eight of the 17 positive samples contained more than one toxin. In addition ten samples contain toxin levels were above legislated limits (aflatoxins) and guidelines (zearalenone and fumonisin) that exist in a few countries. Due to the economic implications and the potential carry-over of toxins from animals to humans through consumption of toxin contaminated milk and meat, maximum tolerance levels for each toxin needs to be legislated for animal feeds.

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