

Canine aflatoxicosis outbreak in South Africa (2011): A possible multi-mycotoxins aetiology

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This study analysed 60 dog food samples obtained from commercial outlets following the 2011 aflatoxicosis outbreak in South Africa. Results obtained from the selected dog food samples revealed that 87% of samples were contaminated with aflatoxins (AFs) (mainly AFB₁ and AFB₂). Amongst these samples, 45 (75%) were above the 20 parts per billion (ppb) set by most countries and 10 ppb regulated by the *Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act* (Act No. 36 of 1947) for South Africa. In addition to AFs, other mycotoxins were also detected in the same samples with fumonisins (FBs) (mainly FB1 and FB2) contaminating 98% of samples with 49 (81.81) above the tolerable limit of 1000 ppb in feedstuff set up by the Federal Drug Agency (FDA) (USA). The FBs mean obtained was 1556 ppb (Table 1) with contamination varying between 5.2 and 4653.8 ppb. Ochratoxin A (OTA) was detected in 41 (68%) of the analysed samples, with a mean value of 13.7 ppb. Amongst these samples, 15 (25%) were above the 20 ppb highest limit set by the Codex Alimentarius standard. Zearalenone (ZEA) was detected in 96% of samples, with a mean value of 354.1 ppb. Thirty-three samples (55%) were above the regulated level 1000 ppb tolerable limit. The recoveries were up to ten times the tolerable daily limits of the FDA and EU. The correlation between mycotoxin findings and clinical signs reported on patients presented for aflatoxicosis led to the conclusion that the outbreak was associated with the presence of other mycotoxins found simultaneously in the analysed samples by additive or synergistic effects.

Introduction

Mycotoxins are fungal metabolites that can reduce the performance and alter the metabolism of livestock and poultry (Wannemacher, Burner & Neufeld 1991). Mycotoxins can be produced by fungi in the field before harvest and may continue to be formed under favourable storage conditions such as temperature, moisture content, and pH following harvesting (Pitt & Hocking 1997). *Aspergillus flavus* is usually found in tropical and semi-tropical climates and produces the carcinogenic hepatotoxic aflatoxin (Klich 2002). It has been confirmed that AFs also affect companion animals. Dogs exposed to AFs contaminated feed develop severe depression, anorexia and weakness, and sudden death may occur (Ketterer *et al.* 1975), with necropsy revealing haemorrhage and liver damage (Arnot *et al.* 2012; Boermans & Leung 2007; Ketterer *et al.* 1975). Chronic aflatoxicosis induces jaundice as the predominant clinical sign and histopathology reveals shrunken livers with extensive fibrosis (Boermans & Leung 2007). Following the 2011 outbreak of dog aflatoxicosis that was recorded in Gauteng Province, South Africa (Arnot *et al.* 2012), dog food samples were collected from retail outlets and analysed for aflatoxins and other mycotoxins.

The aim of the study was to investigate whether other specific mycotoxins could have been involved, in addition to aflatoxins, in the occurrence of the aflatoxicosis outbreak observed in South Africa in 2011.

Materials and methods

Sampling

Sixty randomly selected dog foods were obtained from commercial outlets around Gauteng province and analysed for mycotoxins.

Mycotoxin extraction

In this study, the liquid partitioning or multi-mycotoxin extraction method was used, with minor modifications according to previous publications (Patterson & Robert 1979; Shephard *et al.* 2005; Sydenham, Shephard & Thiel 1992). For mycotoxins identification confirmation, solid phase extraction (SPE) methods were used for FBs extraction based on the method used in previous similar studies (Shephard *et al.* 2005; Sydenham, Shephard & Thiel 1992) for AFs using the immunoaffinity column (IAC) (VICAM) according to the manufacturer's method.

Mycotoxin detection and quantification methods

Mycotoxin detection and quantification of fumonisins (FBs), aflatoxins (AFs), ochratoxin A (OTA) and zearalenone (ZEA) from dog food samples was performed using high performance liquid chromatography (HPLC), model Shimadzu Corporation (Kyoto, Japan). All FBs extracts were re-dissolved in 400 μL of methanol, HPLC grade. For FB₁ analysis, 25 μL of sample or 0.5 $\mu\text{g}/\text{mL}^{-1}$ of standard was mixed with 250 μL of the derivatising agent o-phthalaldehyde (OPA) and injected into the HPLC within 1 min of adding OPA to avoid instability caused by the volatility of the OPA. The mobile phase used was methanol: sodium di-hydrogen phosphate (80:20, v/v) and RF 10AXL fluorescence detector was used with excitation and emission wavelengths set at 335 nm and 440 nm, respectively (Shephard *et al.* 2005). The pH of each extract was adjusted to pH 3.4 and the extract was run isocratically at the rate of 1 mL/min. Fumonisin B₁ and FB₂ were identified by their constant retention time. Quantities were deduced by comparing the peak areas of the standards with those of the samples according to previous studies (Patterson & Robert 1979; Shephard *et al.* 2005). Aflatoxins determination was done according to Reiter, Zentek & Razzazi-Fazeli (2009) as follows: the fluorescence detector RF 10AXL was coupled with a Coring cell (CoBrA cell) (DR Weber Consulting, Germany) as electrochemical cell for the derivatisation of AFM1. The mobile phase was composed of methanol-acetonitrile-water (20:20:60) containing 119 mg of potassium bromide and 100 μL of nitric acid. The excitation was 362 nm and emission was 440 nm with a flow rate of 1 mL/min. For recovery, triplicate runs were done by mixing control samples of milk with 5 ng/mL, 10 ng/mL and 20 ng/mL and run through the immunoaffinity column following the method mentioned above.

The analysis of ZEA was done by fluorescent detection (Abdulkadar *et al.* 2004; Njobeh *et al.* 2009) with some modifications. The excitation and emission wavelengths were optimised at 274 nm and 418 nm, respectively. The mobile phase used was acetonitrile or water (45:55, v/v), pumped at the rate of 1 mL/min. Injection volume per analyte or standard concentrations (5 $\mu\text{g}/\text{mL}$, 10 $\mu\text{g}/\text{mL}$ and 20 $\mu\text{g}/\text{mL}$) was 20 μL . OTA analysis was performed by the fluorescent detection method (Abdulkadar *et al.* 2004; Njobeh *et al.* 2009) with some modifications. The mobile phase consisting of 2% acetic acid/acetonitrile (49:51, v/v) was pumped at a flow rate of 0.8 mL/min. Injection volume of analyte and standard

was 10 μL . OTA was detected at excitation and emission wavelengths of 333 nm and 477 nm respectively.

Results

Results obtained from the selected dog food samples revealed that 87% of samples were contaminated with AFs, mainly AFB₁ and AFB₂ (Table 1). Amongst these, 45 (75%) were above the 20 parts per billion (ppb) set by most countries (Kubo 2012) and the 10 ppb regulated by the *Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act* (Act No. 36 of 1947) (South African Government 2009). The mean concentrations were 248.3 ppb whilst results ranged between 1.2 ppb – 352.7 ppb. In addition to AFs, other mycotoxins were detected in the dog food samples analysed and it is assumed that they could have influenced the effects of AFs. Amongst them, FBs (mainly FB₁ and FB₂) were detected in 98% of samples, with 49 (81.81) above the tolerable limit of 1000 ppb in feed stuff set up by the US Federal Drug Agency (FDA) (Kubo 2012). The FBs mean obtained was 1556 ppb (Table 1) with contamination varying between 5.2 ppb and 4653.8 ppb amongst samples. OTA was detected in 41 (68%) of analysed samples, with a mean value of 13.7 ppb. Amongst these samples, 15 (25%) were above the 20 ppb highest limit set by the Codex Alimentarius (Kubo 2012). ZEA was detected in 96% of samples, with a mean value of 354.1 ppb (Table 1). Thirty-three samples (55%) were above the regulated level of 1000 ppb tolerable limit (Zinedine *et al.* 2007).

Discussion

Dog food contamination by AFs might have been the main cause of death registered in South Africa during the aflatoxicosis outbreak period (Arnot *et al.* 2012). It has been reported from previous outbreaks that dogs exposed to aflatoxin-contaminated food developed severe depression, anorexia and weakness, and sudden death may occur, with necropsy findings showing enlarged livers, disseminated intravascular coagulation, and internal haemorrhage (Ketterer *et al.* 1975; Newberne & Butler 1969). These studies have also revealed that in sub-acute aflatoxicosis, dogs and cats become lethargic, anorexic, and develop jaundice, shrunken liver and extensive fibrosis. These observations correlate with those in the outbreak reported by Arnot *et al.* (2012) and were influenced by the high concentration of AFs noted in this study (Table 1). In addition, AFs have been reported to have an effect on the immune response, to increase cell damage and to induce morphological alterations in cells and reduce

TABLE 1: Mycotoxins distribution in selected dog food obtained from retail outlets in South Africa.

Mycotoxins analysed	Samples (n)	Positive		Samples above set		Means (ppb)	Ranges (ppb)	s.d.
		N	%	Regulatory limits	%			
FBs	60	59	98	49	81.7	1556	5.2–4653.8	1534.2
AFs	60	52	87	45	75	248.3	1.2–352.7	98.2
OTA	60	41	68	15	25	13.7	0.5–53.6	34.5
ZEA	60	58	96	33	55	354.1	2.5–2351.4	362.0

The differences in the mean values amongst the treatment groups are greater than would be expected by chance; there is a statistically significant difference. ppb, parts per billion; FBs, Fumonisin; AFs, Aflatoxins; OTA, Ochratoxin A; ZEA, Zearalenone. p, <0.001

phagocytosis (Richard *et al.* 1993). It is important to mention that AFs contamination in these samples might be explained by the use of ingredients contaminated with AFs producing fungal strains such as *A. flavus* and *A. parasiticus* (Klich 2002; Vargas *et al.* 2007). The quality of ingredients such as maize and groundnuts, oilseeds, cotton seed, millet, sorghum and their derived products used in dog food preparation is important as their moisture content and storage conditions can be determinants for fungal and mycotoxin contamination (Bennett and Klich 2003; Pitt & Hocking 1997, 1999).

In addition to AFs, however, other mycotoxins were detected in dog food samples analysed and their presence could have influenced the clinical signs and symptomatology of dogs presented with aflatoxicosis. Amongst them, FBs (mainly FB₁) were detected in 98% of samples, with a mean of 1556 ppb (Table 1). The detection rate of OTA was 68% of samples analysed, with a mean value of 13.7, whilst that of ZEA was detected in 96% of samples, with a mean value of 354.1 ppb (Table 1). Findings of this study confirm those of several authors who have reported the co-occurrence of mycotoxins in pet food and in other animal feeds (Sedmikova *et al.* 2001; Speijers & Speijers 2004; Stoev *et al.* 2010; Thiel *et al.* 1992).

The presence of FBs, OTA and ZEA in addition to AFs in these food samples might have had an effect on the induction of the outbreak. Fumonisin, known to be produced by *Fusarium verticilloides* (Bezuidenhout *et al.* 1988; Thiel *et al.* 1992; Voss, Smith & Haschek 2007) and also by *F. proliferatum* and *F. nygamai* as well as by *Alternaria alternata* (Marasas 1996; Voss, Smith & Haschek 2007), have been reported to cause toxicity to animals primarily by disruption of lipid metabolism through inhibition-specific ceramide synthase (sphinganine and sphingosine N-acyltransferase), a key enzyme in the pathway leading to formation of ceramide and more complex sphingolipids (Riley & Petska 2005). Fumonisin commonly affect equines by causing equine leukoencephalomalacia (ELEM) (Dutton 1996 2009; Kellerman *et al.* 1990), acute neurotoxicity (Dutton 1996), hepatic necrosis and pulmonary oedema in pigs (Placinta, D'Mello & MacDonald 1999) but not much has been reported on FBs effects on canines. However, the presence of sero-sanguinous ascites, pulmonary haemorrhage and hydrothorax observed in some patients presented at Onderstepoort Veterinary Academic Hospital (OVAH) and reported by Arnot *et al.* (2012) might be explained by the co-occurrence of AFBs and FBs. These observations are similar to those made by Stoev *et al.* (2010) in pigs exposed simultaneously to food contaminated with AFs and FBs. In addition, FBs and trichothecenes have also been implicated in the disturbance of the immune system, digestive system and haemorrhage in animals (Dutton 2009).

Amongst carcasses of patients presented with aflatoxicosis at OVAH, some showed signs of icterus, gastro-enterorrhagia and hepatitis, serosanguinous ascites and serosal petechiation that might also be the effect of exposure to food contaminated with OTA as found in this study. However, OTA might not have had a serious influence because it was

only detected at low concentrations (Table 1) and above the regulated levels (Kubo 2012) in only 25% of samples. OTA seems not to be a major concern for South African crops but it is being detected in samples more and more frequently. OTA is also known to be eliminated via bile and urine and primarily targets kidneys (Hussein and Brasel 2001). Studies have demonstrated that dogs are particularly susceptible when exposed to OTA in food and the kidneys are the most affected organs (Mantle *et al.* 1993; Stoev *et al.* 2011). Companion animal exposure to OTA through feed induces anorexia, prostration and death in cats (Mantle *et al.* 1993). Pathomorphological changes induced by OTA include muco-haemorrhagic enteritis and necrosis of the lymphoid tissues and kidneys (Mantle *et al.* 1993). OTA is partially eliminated via the liver and the kidneys and the relative quantity eliminated depends partly on the animal species, the route of elimination, exposure dosage, the entero-hepatic recirculation and OTA's binding to serum macromolecules (NNT 1991). In addition, it has been reported that OTA toxicity in animal species is dependent upon the 3 α -hydroxysteroid dehydrogenase activity, the isomeric form of its metabolite as well as the ability to excrete as opposed to recycling the glucuronide conjugate (NNT 1991).

Although ZEA is known as an oestrogenic and anabolic mycotoxin with high binding ability to oestrogen receptors (Hussein and Brasel 2001), capable of modulating or disrupting endocrine function and causing induced damage to ovaries, oedema, hyperplasia and general pathological changes in the canine's reproductive system, these signs were not reported in the animals presented for aflatoxicosis. It is assumed that longer exposure would have also induced different symptomatology and clinical signs (Ryu *et al.* 2002). Similar results have been reported by Martins *et al.* (2003), Scudamore *et al.* (1997) and Scussel *et al.* (2006) who, in addition to AFs, found other mycotoxins in pet food respectively in Portugal (OTA), the United Kingdom (FBs) and Brazil (FBs). These results would have influenced the general symptomatology presented by dogs during aflatoxicosis outbreaks (Hussein and Brasel 2001).

A positive answer may be possible for the question of whether the presence of these other mycotoxins (OTA, FBS and ZEA) would have induced and influenced clinical signs in exposed dogs. Studies have been carried out using pigs and human mononuclear cells exposed to OTA, FB₁ and/or AFB₁ (Ryu *et al.* 2002) and on pigs exposed to feed contaminated with OTA and FB₁. Scussel *et al.* 2006 have shown additive effects of these mycotoxins on analysed cells and on the system. In addition, other studies found that the combined administration of AFB₁ and FB₁ resulted in synergistic toxic effects both in the liver and kidney in rabbits (Orsi *et al.* 2007). Creppy *et al.* (2004) also found that the combination of OTA and FB₁ induced *in vivo* and *in vitro* increased cytotoxicity on three different cell-lines, C6 glioma cells, Caco-2 cells and Vero cells. Similar results were obtained in *in vitro* studies using human and pig lymphocytes (Mwanza *et al.* 2009; Ryu *et al.* 2002).

Conclusion

The high levels of dog food contamination with mycotoxins were above the FDA, EU and South African set levels. The presence of other mycotoxins such as FBs, ZEA and OTA in analysed feed samples might most likely have been associated with the 2011 outbreak of aflatoxicosis that caused deaths in South African dogs. However, further *in vivo* and *in vitro* studies are indicated on the effects of concurrent exposure of pets to several mycotoxins in their food. There is a need for a holistic approach during mycotoxin evaluation as more than one toxin can be involved in the appearance of the condition.

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Competing interests

The authors declare that they have no financial or personal relationship(s) which may have inappropriately influenced them in writing this article.

Authors' contributions

M.M. (North West University) was the project leader, carried out the sample analysis and helped prepare the manuscript, R.V.N. (North West University) did the sampling and sample preparation, M.N. (North West University) gave laboratory assistance, did sample analysis and reviewed the manuscript and F.B. (North West University) reviewed the manuscript and gave advice.

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