Toxigenic Potential of Co-occurring Aflatoxin and Ochratoxin A Detected in Poultry feed on *Clarias gariepinus* Larvae

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Abstract: The worldwide contamination of poultry feeds with aflatoxins and ochratoxin A (OTA) independently and in co-occurrence has been reported in several countries. However, there is paucity of information on the co-occurrence of aflatoxins and OTA and their detection by immunoassay in Nigerian poultry feed. Fourty-seven locally formulated poultry samples collected from 13 locations within Southwestern Nigeria were analyzed for total aflatoxins (TA) and ochratoxin A (OTA) using the Immunoassay method. The potential toxicities of the samples were tested by the *Clarias gariepinus* day-old larvae bioassay. Approximately 98.2% samples were positive for TA and OTA with concentrations above the limits of quantitation. The ranges of TA and OTA in the samples were <4.0 µg kg^{-1} to 575 µg kg^{-1} and <2.0 µg kg^{-1} to 14.2 µg kg^{-1} respectively. Toxicity to *C. gariepinus* larvae was concentration dependent and 17, 21 and 7 samples containing the co-occurring toxins showed high, moderate and low toxicities respectively. On the average, 88.9% and 65.5% of the total samples had concentrations above the EU permissible limits for TA in immature and mature poultry feed respectively. The overall contamination risk for TA and OTA in samples in a significant (P = 0.01) decreasing order was: chick mash, broiler finisher, layers mash, broiler starter and growers mash.
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1. Introduction

The worldwide contamination of poultry feeds with aflatoxins and ochratoxin A (OTA) independently and in co-occurrence has been reported in Nigeria (Aletor, 1990), Morrocco (Kichou and Walser, 1993), United States (Schweitzer *et al*., 2001) and Turkey (Nizamlyoglu and Oguz, 2003), and in Argentina (Dalcero *et al*., 1998), India (Thirumala-Devi *et al*., 2002) and Greece (Vlachou *et al*., 2004), respectively. Also is the contamination of independent feeding stuffs involved in poultry feed formulation with aflatoxins and OTA (Aletor, 1990; Atawodi *et al*., 1994; Yazdanpanah *et al*., 2001; Egal *et al*., 2005; Zinedine *et al*., 2007). However, we could not lay hands on any data for the co-occurrence of aflatoxins and OTA and their detection by immunoassay in Nigerian poultry feed.

IARC in 2002 reaffirmed naturally occurring aflatoxins as members of the Group 1 carcinogens while OTA, a frequent natural contaminant of many foodstuffs including poultry feed, eggs and milk (Weidenborner, 2001), was classified as 2B by IARC evaluation in 1993. OTA induces nephrotoxicity, teratogenicity, carcinogenicity and immune-suppression in many animal species including poultry (Huff *et al*., 1974, 1975; Stoev, 1998; IARC, 1993). No animal species is resistant to acute aflatoxicoses. However, animal species respond differently in their susceptibility to chronic and acute aflatoxicoses. Smith and Hamilton (1970) reported that aflatoxin is a very potent hepatotoxin to young broiler chickens whereas Huff and Doer (1981) showed that the targeted effect of the combination of aflatoxins and OTA is on the kidney. The LD50 value of aflatoxins for most species ranges from 0.5 to 10 mg kg^{-1} body weight. Toxicity is influenced by exposure level and duration and environmental factors beside age, health and nutritional status (FAO, 2000).

Due to the above mentioned detrimental effects of these toxins, the European Union (EU) set the maximum tolerable levels for total aflatoxins (TA) in poultry feed and feeding stuffs meant for immature and mature poultry as 10 and 20 µg kg^{-1} respectively: the strictest standards for TA in the world (EC, 2003). Considering the need for appropriate regulation of mycotoxins in the Nigerian feed industry and the serious damaging effects caused by the dietary toxin combinations in poultry this research aimed at

surveying some states in Southwestern Nigeria for locally formulated poultry feed, detecting and quantifying TA (AFB_1 + AFB_2 + AFG_1 + AFG_2) and OTA contamination in the feeds. The potential toxicity of the contaminated samples was evaluated using *Clarias gariepinus* larvae bioassay.

2. Materials and Methods

2.1 Sample Collection

Five categories of poultry feed were collected between April and September, 2009 and examined in this study. The feed categories were chick mash, growers mash, layers mash, broiler starter and broiler finisher. The locations where the local poultry feed rations were formulated were surveyed and samples collected according to the categories above. The bags containing the raw materials (maize kernels, soybean meal, wheat offals and groundnut cake) from which the samples were formulated were stacked on each other on the very dusty cemented floors of the warehouses. The bags had been in store for at least 4 months prior to collection time. These conditions were enough to serve as avenue for entry of toxigenic fungi, their growth and consequent toxin liberation. For each sampled bag of feed (50 kg), three identical sampling sizes (1 kg each) from three different points of the bulk feed (top, middle and bottom) were taken at same time and mixed thoroughly to form the sample lot (3 kg per sample lot) for analysis. From the sample lot, 1 kg representative sample was taken and ground into fine powder with a Waring blender. Ground samples were stored at -4 ^oC until subsequent extraction and analysis.

Samples were collected from 13 locations in four states of Southwestern Nigeria after a preliminary survey. The states and representative locations were Lagos (Ikorodu, Iyana-Ipaja and Okoko), Ogun (Ijebu-Ode, Shagamu, Abeokuta and Ikenne), Oyo (Ibadan, Oyo, Moniya and Apata) and Osun (Ife and Oshogbo). Representative samples were labeled prior to grinding and analysis of samples for TA and OTA (October 2009 – May 2010).

2.2 Chemicals and Kits

ACS-grade methanol was purchased from Sigma-Aldrich (Germany). Validated Immunoassay [direct competitive Enzyme-linked Immunosorbent Assay (ELISA)] kits for Total Aflatoxins (B_1, B_2, G_1, G_2) and Ochratoxin A quantitation were purchased from Romer Labs® (Singapore).

2.3 Toxin Extraction and Quantitation by ELISA

A 20 g analytical sample was taken from each comminuted representative sample into a clean jar. To this jar 100 mL of 70% (v/v) methanol extraction solution was added and the jar sealed. The jar was shaken vigorously in a rotatory shaking incubator (150 rpm for 3 min) and then allowed to stand for 5 min. The top layer was filtered off into a clean glass bottle through 2-folds of Whatman #1 filter paper and the pH of the filtrate was tested. The extraction procedure was applied for both TA and OTA extraction from the feed matrix bearing in mind the need to pay full attention to sample preparation even if it is impossible to attain 100% certainty in determining mycotoxin concentration in a bulk lot (Whitaker, 2006).

Quantitation of TA (AFB_1 + AFB_2 + AFG_1 + AFG_2) and OTA in µg kg^{-1} were carried out following the procedures outlined in 96-well commercial AgraQuant® Total Aflatoxin Assay 4/40 kits and 96-well commercial AgraQuant® Ochratoxin Assay 2/20 kits respectively. The screened samples were read using the EL301 microwell reader (BIO-TEK® Instruments, Inc) at absorbance OD of 450 nm and 630 nm differential filters. The results were calculated using the Romer® Log/Logit spread sheet and interpreted with the Log/Logit regression model. The Limit of quantitation (LOQ) and Range of quantitation (ROQ) for TA were 4 µg kg^{-1} and 320 µg kg^{-1} respectively and 2 µg kg^{-1} and 40 µg kg^{-1} respectively for OTA. Repeated dilutions of 5 – 20 µg kg^{-1} were made on samples to extrapolate quantitation value above 320 µg kg^{-1} for TA.

2.4 Toxicity Assay

The Brine shrimp (*Artemia salina* L.) larvae bioassay for mycotoxin toxicity as described by Korpinen (1974) was modified slightly in the choice of the test organism and conditions. Day-old larvae of freshwater African catfish, *Clarias gariepinus*, otherwise called fingerlings were used in our study. The choice of *C. gariepinus* larvae was based on availability as well as similar morphology and physiology of *Clarias* to *A. salina*. Moreover, Gbore *et al*. (2010) reported the application of *Clarias* in mycotoxicity studies using Fumonisin B_1 as target toxin.

Actively motile day-old larvae were maintained under constant purified oxygen in clean oxygen bags filled with 150 mL tap water containing 0.002% (w/v) NaCl. Treatments were set up in a completely randomized design such that each oxygen bag contained 10 larvae. This was replicated in four sets per treatment (feed sample) to give a total of 200 oxygen bags and 2000 larvae. The fingerlings in each bag labeled against each test feed sample were exposed to 100 mg of the test feed sample. Positive control bags (P_1, P_2, P_3) contained larvae that were exposed to 5 µg L^{-1} pure aflatoxin, P_1; 5 µg L^{-1} pure ochratoxin, P_2; a mix of 2.5 µg L^{-1} each of pure aflatoxin and OTA, P_3. The concentration ratio of individual aflatoxin types in the 5 µg L^{-1} TA was 5:1:3:1 for AFB_1, AFB_2, AFG_1 and AFG_2 respectively. Negative control bags (N_1 and

N_2) contained larvae exposed separately to the two feed samples (L3 and F5) that contained < 4 µg kg^{-1} TA and < 2 µg kg^{-1} OTA respectively.

The exposed larvae were incubated at 25 ^oC for 24 h under oxygen. The number of dead larvae was recorded after the incubation period. Total loss of locomotive action of larvae was interpreted as death. The total number of fingerlings per bag was counted after freezing the bags to kill the survivors at -10 ^oC for 6 h. Results were recorded as High toxicity (H) when x < 75% mean mortality of larvae, Moderate toxicity (M) for 50 < x ≤ 75% mean mortality of larvae, Low toxicity (L) for 25 ≤ x ≤ 50% mean mortality of larvae and No toxicity (NT) when x = 0% mean mortality of larvae (Youssef, 2009).

2.5 Statistical Analysis

The results from individual analyses of samples are presented in µg kg^{-1} (ppb). The individual and combined mycotoxin concentrations were evaluated for feed categories for the comparison of the exposure risk of poultry to the toxic feed. The non-parametric Wilcoxon rank sum test (WRST) of Gad and Neil (1982) was used for this comparison at P = 0.01.

3. Results

A total of 47 samples were collected and analyzed for TA and OTA in this study. TA and OTA were detected at varying levels in all of the feed samples analyzed in this study except L3 (N_1) and F5 (N_2). These two samples (L3 and F5) had either TA or OTA concentration below their LOQs and they were from Ife and Apata respectively. All the feed types collected contained different quantities of the combinations of corn, groundnut cake, soybean meal, wheat offals, fish meal, sodium chloride and different kinds of additives including premixes, ferrous tabs, etc. Table 1 shows that the order of TA and OTA contamination in samples decreased in the order: chick mash, broiler finisher, layers mash, broiler starter and growers mash (P = 0.01). The summary of the analysis of poultry feed types for TA and OTA (Table 1) show that the growers mash had the lowest TA range of contamination (6.1 – 95.4 µg kg^{-1}) with 28.0 µg kg^{-1} as mean value while chick mash had the highest contamination range of 25.1–575 µg kg^{-1} and mean of 268.1 µg kg^{-1}. All feed categories had TA contamination of 100% except the layers mash which recorded 92.3%. The broiler finisher feed had the lowest range of OTA contamination (<2.0 – 14.2µg kg^{-1}) with mean value of 10.9 µg kg^{-1} while the range for chick mash samples was the highest (11.4 – 13.6 µg kg^{-1}). Only broiler finisher feed had a percentage contamination level lower than 100 (92.3%).

Table 1. Analysis of Total Aflatoxins and Ochratoxin A profiles in local poultry feed

Parameter	Poultry feed types				
	Chick mash *n = 8*	Growers mash *n = 9*	Layers mash *n = 10*	Broiler starter *n = 10*	Broiler finisher *n = 10*
Total Aflatoxin					
Range (µg kg^{-1})	25.1 – 575	4.7 – 95.4	<4 – 146.2	4.7 – 107.7	4.1 – 124.9
Mean value	268.1	28.0	49.5	33	63.9
% Positive	100	100	92.3	100	100
Ochratoxin A					
Range (µg kg^{-1})	11.4 – 13.6	6.1 – 14	3.2 – 13.9	6 – 13.7	<2 – 14.2
Mean value	12.5	11.0	11.2	10.5	10.9
% Positive	100	100	100	100	92.3

n = number of samples assayed

The independent analysis for TA and OTA contamination levels in the five categories of poultry feed, mean percentage mortality of the exposed larvae and the toxicity levels are presented in Tables 2 – 6. All the eight chick mash samples were contaminated with TA far above the EU permissible limits for immature poultry (Table 2) while the toxicity level studies expressed from the mean percentage mortality values showed that only 25% (2/8) of the samples that violated the EU limit caused moderate toxicities to *C. gariepinus* larvae. The other six samples where highly toxic. Positive control bags (P_1 and P_3) exhibited high toxicities whereas P_2 which contained 5 µg L^{-1} pure OTA showed low toxicity to the day-old larvae.

The data for the growers mash samples (Table 3) showed that only 44.4% of the samples had TA concentration levels above EU limits for mature poultry: an insignificant value (P = 0.01) as compared to the samples of other feed categories that violated the limits for mature birds. The toxicity levels of the violating samples range from moderate to high toxicity. From Table 4, 80% of the layers mash samples violated the EU regulation of TA levels in mature poultry feed. One particular sample coded as L3 (N_1) had TA level below the LOQ (4 µg kg^{-1}) for the method used. Toxicity levels of the violating samples ranged from moderate to high toxicities.

Table 2. Total Aflatoxins (TA) and Ochratoxin A (OTA) concentrations in chick mash samples and *C. gariepinus* toxicity

	Positive controls (n = 3) and Chick mash samples (n = 8)										
Analysis	P_1	P_2	P_3	C1	C2	C3	C4	C5	C6	C7	C8
$^+$TA	-	-	-	427.5*	230*	251.8*	470*	575*	25.1*	35*	130.3*
$^+$OTA	-	-	-	12.5	13.6	11.4	11.9	11.6	12	13.6	13.3
Mean % mortality	85	32.5	87.5	95	87.5	87.5	100	100	65	70	87.5
Toxicity	H	L	H	H	H	H	H	H	M	M	H

$^+$TA and OTA values are in (µg kg^{-1})
*samples above EU Permissible Limits for TA in immature poultry feed (% samples = 100%)
P_1: Positive control 1, P_2: Positive control 2, P_3: Positive control 3
H: High toxicity, M: Moderate toxicity, L: Low toxicity

Table 3. Total Aflatoxins (TA) and Ochratoxin A (OTA) concentrations in growers mash samples and *C. gariepinus* toxicity

	Growers mash samples (n = 9)								
Analysis	G1	G2	G3	G4	G5	G6	G7	G8	G9
$^+$TA	18.3	25.7*	4.7	6.1	18.4	18.1	25.6*	95.4*	40*
$^+$OTA	6.1	12.1	10	10	9.7	14	13	11.8	12.6
Mean % mortality	62.5	67.5	25	27.5	67.5	42.5	65	77.5	75
Toxicity	M	M	L	L	M	L	M	H	M

$^+$TA and OTA values are in (µg kg^{-1})
*samples above EU Permissible Limits for TA in mature poultry feed (% samples = 44.4%)
H: High toxicity, M: Moderate toxicity, L: Low toxicity

Table 4. Total Aflatoxins (TA) and Ochratoxin A (OTA) concentrations in layers mash samples and *C. gariepinus* toxicity

	Layers mash samples (n = 10)									
Analysis	L1	L2	N_1	L4	L5	L6	L7	L8	L9	L10
$^+$TA	20.8	20.5	<4	30.5	20	33	109.1	80	31.1	146.2
$^+$OTA	6.2	13.9	3.2	11.5	13.1	12.3	13.1	12.9	13.5	12.2
Mean % mortality	65	62.5	0	70	65	70	80	77.5	62.5	87.5
Toxicity	M*	M*	NT	M*	M	M*	H*	H*	M*	H*

$^+$TA and OTA values are in (µg kg^{-1})
*samples above EU Permissible Limits for TA in mature poultry feed (% samples = 80.0%)
N_1: Negative control 1 (L3)
H: High toxicity, M: Moderate toxicity, NT: No toxicity

Table 5. Total Aflatoxin (TA) and Ochratoxin A (OTA) concentrations in broiler starter samples and *C. gariepinus* toxicity

	Broiler starter samples (n = 10)									
Analysis	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
$^+$TA	76*	4.7	20.1*	6	20*	17.8*	22.2*	45*	107.7*	10.2*
$^+$OTA	13.2	2.8	13.1	6.5	13.3	13.7	12.8	11.4	13.1	5.5
Mean % mortality	75	25	60	27.5	60	60	60	72.5	77.5	32.5
Toxicity	M	L	M	L	M	M	M	M	H	L

$^+$TA and OTA values are in (µg kg^{-1})
*samples above EU Permissible Limits for TA in immature poultry feed (% = 80%)
H: High toxicity, M: Moderate toxicity, L: Low toxicity

Table 5 shows that 80% of the 10 broiler starter samples had concentrations above EU limits for immature poultry. The samples with levels above EU limits also showed moderate to high toxicities. Only 70% of the broiler finisher samples violated TA regulation in mature poultry feed by EU (Table 6). One sample known as F5 (N_2) had

OTA concentration below the LOQ (2 µg kg^{-1}) and TA concentration of 4.1 µg kg^{-1} thereby producing no toxicity to the larvae. Toxicity of the feed samples to *C. gariepinus* larvae was concentration dependent.

Table 6. Total Aflatoxin (TA) and Ochratoxin A (OTA) concentrations in broiler finisher samples and *C. gariepinus* toxicity

Analysis	Broiler finisher samples (n = 10)									
	F1	F2	F3	F4	N_2	F6	F7	F8	F9	F10
$^{+}$TA	79*	80*	75*	5.7	4.1	120.7*	18.3	56*	124.9*	75.2*
$^{+}$OTA	12.7	12.1	11.7	4.8	<2	13.2	10.4	14	14.2	13.9
Mean % mortality	77.5	77.5	80	25	0	87.5	62.5	75	87.5	77.5
Toxicity	H	H	H	L	NT	H	M	M	H	H

$^{+}$TA and OTA values are in (µg kg^{-1})
*samples above EU Permissible Limits for TA in mature poultry feed (% = 70%)
N_2: Negative control 2 (F5)
H: High toxicity, M: Moderate toxicity, L: Low toxicity, NT: No toxicity

4. Discussion

Subsistence poultry farming has become the major source of livelihood in some parts of Nigeria with the Southwestern and Eastern regions having more of these farms. Reports from the local farmers indicated the use of low quality grains which do not meet human consumption standards in poultry feed formulation. These grains showed moldiness, discolorations and numerous cracking from insect infestation. Quality poultry feed is necessary for the maintenance of physiological functions and animal defense systems against diseases and parasites. Traditionally feed quality has been specified on basis of the nutritional value of every individual feed component (Fink-Gremmels, 2004). This may not be true microbiologically as recorded in this study since it is expected that quality feed should be highly nutritious and at the same time free of any form of hazardous contaminants such as microbes, toxins and/or heavy metals.

The worldwide co-occurrence of aflatoxins and OTA in poultry feed formula and feeding stuffs as documented earlier has been upgraded for Nigeria by our study although TA occurred in higher concentrations than OTA in every feed sample. The low quantities of OTA present in the samples may be due to a low occurrence of OTA-producing fungi, unfavorable conditions for expression of OTA biosynthetic genes if toxigenic fungi are present or other fungal interactions and environmental conditions (Schmidt-Heydt *et al*., 2007; Wagacha and Muthomi, 2008). OTA contamination on its own may not necessarily pose any serious harm since it occurred in low quantities for all samples. But the high values obtained for TA in the samples with levels above EU limits and the percentage violating sample per feed type is alarming. It is logical to suggest that the individual feed components may have originally been contaminated with somewhat moderate levels of these toxins bearing in mind that cereal- and oil-based ingredients are suitable substrates for fungal growth and mycotoxin liberation. This is in line with the suggestions of Egal *et al.* (2005) and Zinedine *et al.* (2007) that toxin contamination of feed may be due to the presence of contaminating toxigenic fungi in individual feeding stuffs (corn, groundnut cake and wheat) or in finished product, feed processing environments and/or storage environs and conditions. This high level of TA may therefore pose a great risk to the poultry industry in terms of fowl death, reduced income, low egg production and quality, and administrative tension.

It is on record that the co-occurrence of OTA with aflatoxins could lead to higher degree toxicity in poultry (Huff *et al*., 1983; Petzinger and Ziegler, 2000). The day-old larvae may have died following the proposed cascade of events by Eaton and Gallager (1994), Creppy (1995), Riley and Norred (1996) and Benford *et al.* (2001) for the toxicity of co-occurring mycotoxins. The events may have progressed from metabolic activation of the toxin through DNA modification or inhibition of protein synthesis or altered membrane permeability and calcium transport disruption to cell deregulation, and cell death which was expressed in our study by the total loss of locomotive action of the larvae.

The observation from Table 2 where the positive control with 5 µg L^{-1} pure OTA (P_2) showed low toxicity as compared to the high toxicity of the other positive controls corresponds with the findings of Sokol *et al*. (1988) and this confirms that OTA alone even at relatively high concentrations is less toxic to this species of *Clarias*. Sokol et al. reported that OTA is less toxic in independent administration but when in

combination with aflatoxins exhibits a very high toxicity level that is dose dependent. The moderate to high toxicities observed in test experiments having moderate combinations of TA and OTA could have resulted from synergistic and potentiated effects of both toxins. Huff and Doerr (1981) reported a synergistic effect in a study of the combined effects of AFB_1 and OTA in broiler chicken. In further studies carried out by Huff *et al.* (1983) and Huff *et al.* (1988) they noticed that the toxic effects of aflatoxins and OTA combinations when compared to the toxicity expressed by some other mycotoxin combinations in poultry and pigs was found to be the most toxic. Similarly Sedmikova et al. (2001) reported that the co-occurrence of OTA with AFB_1 in same substrate even at very low concentrations was capable of inducing a potentiated mutagenic activity of AFB_1 in organisms as assayed by the *Salmonella typhimurium* Ame's test. Therefore our data confirm previous reports on combined toxicity of OTA and aflatoxins thereby adding to the data.

From the toxicity studies also, we are suggesting the likelihood that the type of aflatoxin present and the specific quantities in which they occur in a sample goes a long way in influencing the toxicity level induced when combined with OTA. This is deduced from the case of sample G6 which had TA and OTA concentrations of 18.1 and 14 µg kg^{-1} respectively. Considering these values which are almost similar to those of G1 and G5 one would have expected a moderate toxicity effect. Since G6 caused a low toxicity effect it could be that this sample contained more of the less toxic aflatoxin types such that they could not synergize with OTA to induce a moderate form of toxicity as we know from literature the decreasing order of toxicity in aflatoxin types (AFB1, AFG1, AFB2, AFG2) (Wogan *et al.*, 1971). This therefore calls for further analysis of the samples to determine the specific aflatoxin types and ratios.

Conclusively our study has shown that aflatoxin contamination of locally formulated poultry feed is very high in Nigeria unlike ochratoxin A contamination. We have also exposed the impending danger of poultry toxicity upon dietary consumption of aflatoxin and OTA in combination and suggested that toxicity may be concentration dependent for this duo depending on the type of aflatoxin present and the proportion of occurrence. This is the first report of the co-occurrence of aflatoxins and ochratoxin A in poultry feed from Nigeria by the Immunoassay method. Further investigations are underway towards the analysis of the poultry feed samples for other contaminating toxins, isolation and characterization of the mycotoxigenic fungi present.

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