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AFLATOXINRISKINDAIRYPRODUCTIONASSESSMENTOFDAIRYCATTLEFEEDCONTAMINATIONBYASPERGILLUSFLAVUSANDA.PARASITICUSINBOTHCONVENTIONALANDTRADITIONALDAIRIES

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Aflatoxin Risk in Dairy Production: Assessment of Dairy Cattle Feed Contamination by *Aspergillus Flavus* and *A. Parasiticus* in both Conventional and Traditional Dairies

G. K. Omeiza [α], J. Kabir [σ], J. K. P. Kwaga [ρ], C. N. Kwanashie [ω], M. Mwanza [¥] & L. Ngoma [§]

Abstract- Contamination of dairy cattle feeds by aflatoxigenic Aspergillus group, poses public health challenges as a result of high chances of aflatoxicosis. In this study, therefore, dairy cattle feed samples (n=144) collected from both conventional and traditional dairies were examined for the presence of aflatoxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* using microbiological and molecular techniques. Fungal Colonial counts (CFU) were determined, and the mean CFU/g of the feed samples was 3.8 ± 0.47. A Significant number of the feeds, 86 (59.7%) showed positive contamination level, out of which 55.8% and 18.6% (representing 33.3% and 8.3% of the total dairy cattle feed samples collected) were contaminations due to *Aspergillus flavus* and *Aspergillus parasiticus* respectively. All the 64 isolates of the *A. flavus* and *A. parasiticus* were examined for aflatoxin producing abilities under a long UV light (365 nm). Aflatoxin production levels were quantitatively determined using ELISA technique and 16 isolates representing 25.0% of the total isolates; in the ratio of 3:1 respectively, showed a varied level of production of aflatoxins. Distribution of the aflatoxigenic strains was highest amongst the feeds collected from the traditional Fulani dairy herds showing a prevalence of 8 (50.0%) of the total identified aflatoxigenic strains and lowest, 2 (12.5%) among the conventional dairies. The observed effect of the *aflR* gene, suggests that it was capable of suppressing other structural genes such as O-methyltransferase *(omt),* vericocysteine *(ver)* and norsolorinic *(nor),* involved in the biosynthesis of aflatoxins. Both microbiological and molecular studies identified intermediary and potential aflatoxin-producing (IPAP) strains of the *Aspergillus flavus* and *Aspergillus parasiticus*. The occurrence of *Aspergillus flavus* and *Aspergillus parasiticus* in dairy cattle feeds was significantly higher ($p < 0.05$) compared with the low level of their corresponding aflatoxigenic strains. Traditional dairies were found to constitute public health risk about the presence of the significant number of the aflatoxigenic strains as feed contaminants.

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I. Introduction

Aflatoxins are toxic and carcinogenic metabolites produced by some strains of *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius.* These species of fungi are commonly found in the environment, foodstuffs and animal feed, but its population increases during hot-humid weather (Hedayati *et al*., 2007). Aflatoxins (AF) are found in grains that have been produced under stressed conditions (Naidoo *et al*., 2002). *A. flavus* grows and develops faster under a relative humidity of 85%, a moisture content of the surface of about 30%, temperature of 25°C and a suitable substrate (Tvrtković, 2006). Thus, drought, heat, insect, nematode and fertilizer stress, all promote high levels of AF production. However, the mere presence of these fungi, may not depict toxigenicity as not all strains can produce aflatoxins, and this explains the need for ascertaining their aflatoxin producing abilities.

In Nigeria, Traditional Fulani Dairy Herds (TFHs) are responsible for about 80% of the total milk production (Ajala, 2004). These products are frequently purchased in significant quantities to boost the low level of production by the few available conventional dairy farms. Feeds from the traditional Fulani dairies may suffer heavy contamination by *Aspergillus flavus* due to poor husbandry practices. Such complementary practices between the conventional dairies and TFHs may pose serious public health risks particularly in situations where the status of the contaminating fungi is uncertain. The above critical issues have informed the basic needs to evaluate the aflatoxin-producing capabilities of the isolates of the *A. flavus* and *A. parasiticus* commonly associated with the dairy cattle feeds.

II. Materials and Methods

a) Feed sampling

Feed samples were obtained as fresh and preserved (stored) samples (where applicable) from 6 selected commercial and institutional farms on the one hand, and four traditional Fulani cooperative herds on

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the other. Polytene bags and metal probes were purchased and sterilized for sample collections from troughs and stores respectively. In the case of stored samples, systematic random sampling technique was adopted. Assuming an imaginary diagonal line, bags of feed were randomly selected at intervals of three for probing at different points to pool an estimated representative sample of averagely 40g each. In the case of unpreserved feed, two feeding troughs containing the feed were examined among others in the milking parlor. Collected feed samples were pooled to make one representative feed sample per farm/herd. Sampling was carried out at weekly intervals until a total pooled feed samples reached 144. For the ethical reason, names of farms and cooperative herds used in this study were identified as Farm A (NP), Farm B (DC), Farm C (YS), Farm D (CG), Farm E (JM), Farm F (GG). Other farms comprising of Traditional Fulani dairy cooperatives (FH) were also identified as EM, JN, AL, and JE.

b) *Cultural isolation of Aspergillus flavus and Aspergillus parasiticus*

Feed sample preparation was carried out in line with the methods reported by Makun *et al*., (2010) and Udom *et al*., (2012). Forty gram of feed sample was collected from each farm and homogenized out of which 1g was taken and prepared as one fold dilution in a test tube using 9 ml sterile water. Using a sterile syringe, 1 ml of the feed suspension was dispensed on to a sterile Saboraud Dextrose Agar (SDA) medium. A sterile spreader was employed to gently and evenly spread the dispensed feed suspension. The preparation was incubated at an ambient temperature of 28°C in a relatively dark place for 3-5 days. Colonies which appeared greenish yellow with powdery texture having the reverse side pale to yellow were treated as suspects (Mycology-Critique, 2004). Suspected colonies of *Aspergillus* spp were counted and presented as Log10 CFU/gram of feed according to the method of Udom *et al*. (2012). Pure cultures of the colonies were obtained after repeated isolation and maintained as stock cultures in water culture technique and kept at ambient temperature according to the method reported by Larone (1995).

c) *Identification of the A. flavus and A. parasiticus using phenotypic techniques*

Identification of *Aspergillus flavus* was carried out according to the method reported by James and Natalie (2001) and Bandh *et al*. (2012) using microscopic and macroscopic morphologic techniques respectively. Primary macroscopic morphological studies were carried out on SDA while Czapek Dox Agar and Rose Bengal agar served as differential media. Aflatoxigenic potential of the *Aspergillus* spp under study utilized desiccated coconut impregnated neutral red agar, sometimes referred to as, *neutral red desiccated coconut agar (NRDCA)* as described by Atanda *et al*., (2011). Fluorescence characteristics of produced aflatoxin around each colony of *Aspergillus* were observed and categorized into very strong fluorescence, strong fluorescence, weak fluorescence and non-fluorescence (negative samples). Microscopic studies, on the other hand, were carried out using lactophenol staining as previously described (James and Natalie 2001; Ibrahim and Rahma, 2009).

d) *Quantitative determination of the associated aflatoxin B1 on NRDCA using HPLC*

Aflatoxin B1 Content: About 2 g of the positive NRDCA agar sample was extracted and homogenized simultaneously for 10 min in a homogenizer with 6 ml of methanol-water mixture (4+1). The mixture was spinned by centrifugation for ten minutes at 3500 round per minute. An aliquot of 100 µl of the supernatant was diluted with 700 µl of phosphate buffer, and the resultant solution used for the determination of AFB1.

AFB1 Clean-up procedure and determination

A 5ml aliquot of the extract was added to 14 ml of phosphate buffered saline (1 x PBS) solution (8.0g NaCl, 1.2 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2g KCl, dissolved in 990 ml purified water) and pH adjusted to 7.0 with HCl. The diluted filtrate (19 ml) which is equivalent to 1gram of the sample was passed through the Aflatest® IAC at a flow rate of 2 ml per minute to enable the aflatoxin captured by the antibodies present in the column. After that, the column was cleansed with 20ml of 1 x PBS at a flow rate of 5ml per minute to remove the unbound material, until air passed through the column. Eluate-containing aflatoxins, was eluted from the column with 1 ml of 100% methanol at a flow rate of 1 drop per second and 1 ml of water passed through the column and collected in the same vial to give a total of 2 ml. The eluate (AFs extract) collected in the amber vials, was evaporated to dryness with stream of nitrogen gas at 50°C and stored at +4˚C. The resulting dry extracts were subsequently dissolved in 500 µl of HPLC grade acetonitrile. The sample extracts were analyzed at a flow-rate of 1 ml per minute (min^{-1}) retention times. The analysis of the extract of aflatoxin involved the coupling to a detector a coring cell (CoBrA cell) (Dr. Weber Consulting, Germany) as an electrochemical cell for the derivatization of aflatoxins; using methanol/Acetonitrile/ Water (20/20/60, v/v/v) containing 119 mg of potassium bromide (KBr) and 350ul of nitric acid (4M HNO_3) as mobile phase.

e) *Polymerase Chain Reaction Methods Used to Detect the Strains of Aspergillus flavus*

Specific PCR was carried out to increase the sensitivity of gene identification. All the molecular methods used in this study were harmoniously carried out under the same conditions of PCR.

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i. *Fungal DNA extraction*

The fungal genomic DNA was extracted using Fungal/Bacterial DNA extraction kit (Zymo Research Corporation, Southern California, USA) according to the manufacturer's instructions. The DNA of 5-day old cultures of the isolates grown on PDA plates was extracted. A sterile wire loop was employed to harvest the fungal mycelia, by scrapping the agar surface using sterile wire loop to obtain about 200 mg of mycelia.

ii. *Fungal identification to the strain level*

The internal transcribed spacer (*ITS*) region homologous to fungi was amplified by PCR using the primer set: FF2; 5'-GGT TCT ATT TTG TTG GTT TCT A-3' (forward) and FR1; 5'-CTC TCA ATC TGT CAA TCC TTA TT-3' (reverse) designed by Zhou *et al*. (2000) which encodes an amplicon size of 674bp. The identification of the genus Aspergillus was achieved through amplification of the intergenic spacer region (IGS) of the fungal DNA homologous to the genus *Aspergillus* using a primer set: *Asp*-F, 5'-CGGC CCTTAAATAGCCCGGTC-3'; *Asp*-R, 5'-ACCCCCCTGAGCCAGTCCG-3' encoding an amplicon size of 500 bp described by Willem *et al*. (1994). The IGS is located between V7 and V9 regions of the *18S rRNA* (White *et al*., 1990; Willem *et al*., 1994; Latha *et al*., 2008). Identification of *Aspergillus flavus* utilized specific primer (*Fla*-F., 5' -GT A GGG TTC CT A GCG AGCC-3'; *Fla*-R., 5'-GGA AAA AGA TTG ATT TGCG-3') encoding an amplicon size of 500bp, described by Gonzalez-Salgado *et al*. (2008) to identify certain flanking gene fragment *(Fla)* specific to *A. flavus,* located within the highly variable portion of the internally transcribed spacer regions, *ITS*.

iii. *PCR Reactions*

Individual PCR reactions contained 4µl of DNA (12-116 ng /µl) template which was mixed with 25 µl master mix (Taq DNA polymerase (Fermentas Life Science, Lithuania), dNTPs, $MgCl_2$ and reaction buffers), 1 µl of the primer i.e. Reverse (0.5 µl), Forward (0.5 µl) and 20 µl of nuclease-free water to make up a reaction volume of 50 µl. A negative control was also prepared to contain all the reagents except the DNA. The PCR experiment was carried out in eppendorf tubes placed in a C1000 Touch™ thermocycler (Bio-Rad, USA) with the following reaction conditions: initial denaturation temperature of 95°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute. Primer reannealing temperature was held at 58°C for 45 seconds and extension at 72°C for 1.5 minute .The PCR was finally extended for 10 minutes at 72°C and held at 4°C until samples were retrieved.

iv. *Molecular differentiation between A. flavus and A. parasiticus*

The IGS, *aflJ-aflR,* enclosing the aflatoxin biosynthetic gene was amplified using the primer sequence: IGSF,5′AAGGAATTCAGGAATTCTCAATTG3′; IGSR,5′GTCCACCGGCAAATCGCCGTGCG-3′ previously reported (Ehrlich *et al*., 2003, 2007) that correspond to a PCR product of 674 bp which discriminates between *A. flavus* and *A. parasiticus*. Restriction site analysis (PCR-RFLP) of the PCR products of the IGS was carried out to achieve this. The amplified PCR products were subjected to endonuclease restriction enzyme digestion using *Bg III* (Zymo Research Corporation, Southern California, USA) in a total reaction volume of 40 µl containing 15 units of enzyme, 4 µl of buffer, 15 µl of PCR product, and Ultrapure water up to 40 µl. The reaction mixture was incubated at 37°C for 3 h. Then the resulting fragments were separated by electrophoresis on a 2% w/v agarose gel for 1 h 45 min at 100 V.

v. *In vitro detection of genes that encode aflatoxin production*

In this study, the effects of 3 structural and 1 regulatory gene were studied to evaluate aflatoxin-producing capabilities among the isolated *Aspergillus flavus.* These include: Norsolorinic reductase *(nor),* O-methyl transferase *(omt),* Vesicolorin dehydrogenase *(ver)* and Aflatoxin regulated gene *(aflR).* The Primers used have been previously described (Geisen, 1996; Criseo *et al*., 2001; Gonzalez-Salgado *et al*., 2008; *Latha el al.,* 2008; Rashid et a1., 2008).

vi. *Gel electrophoresis of PCR products*

Agarose gel DNA electrophoresis was performed according to the method previously described (Saghai-Maroof *et al*., 1984). Briefly, the PCR product (8 µl) mixed with 6 µl of loading dye was pipetted slowly into each of the wells in the gel with a sterile micropipette. Care was taken not to cross-contaminate the wells. A 6 µl of the molecular marker also referred to as Gene Ruler (1-kilo base (kb) DNA ladder (Fermentas Life Science, Lithuania) was pipetted into the first and last wells. The chamber was closed and ran at 400 V, and 100 mA for 30 minutes and DNA fragments were viewed by removing the gel slab from the tray and placed on a UV transilluminator, the Geldoc™ MP imaging system (Bio-Rad Laboratories, California, USA).

f) Statistics

Data generated were subjected to Fisher's Exact Test using SPSS statistical software of version 20.0. Null hypotheses were analyzed and the statistical level of significance was fixed at p-value less than 0.05.

III. Results

a) The Occurrence of Aspergillus species in dairy cattle feed

Out of the 144 dairy cattle feed samples collected across different dairy farms and herds and tested, 86 (59.7%) yielded *Aspergillus* species. Of these 86 isolates of *Aspergillus* spp from dairy feeds, 48 (55.8%) and 16 (18.6.7%) representing 33.3% and 8.3%

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were *Aspergillus flavus* and *A. parasiticus* respectively as shown in Table 1. Out of the 64 isolates of *A. flavus* and *A. parasiticus* isolated, 16 (25.0%) were aflatoxigenic in the ratio of 3: 1 respectively (Table 1). Other *Aspergillus* species isolated in this study were distributed in the following proportions: *Aspergillus fumigatus* (7.9%), *A. tamarii* (1.8%), *A. niger* (11.4%) and *A. vesicolor* (4.5%). The occurrences of these are presented in Table 2 as Log_{10} CFU (logarithmic value for colony forming unit) per gram of analyzed feed samples in accordance to WHO pattern of reporting as reported by Udom *et al.* (2012). A mean colonial count of (μ=3.8) Log_{10} CFU/g was determined. There was no statistically significant difference ($P>0.05$) in Aspergillus counts (Log_{10} CFU/g) between fresh and stored feed samples, even though, apparently higher CFU values were noticed among the stored feeds of all dairy feed types analyzed in this study. *Aspergillus flavus* was found predominant (4.5 CFU/g of feed) among the feeds fortified with concentrates than any other feed types (Table 2).

b) Contamination of dairy cattle feed by aflatoxigenic strains of A. flavus

The 64 isolates of *A. flavus* and *A. parasiticus* were examined for aflatoxin production under a long UV light (365 nm), and 16 (25.0%) comprising of 12 (18.75%) *A. flavus* and 4 (6.25%) *A. parasiticus* representing 8.3% and 2.8% respectively (Table 1), showed varying degrees of aflatoxin production (Table 3). The remaining 48 (75.0%) isolates of *A. flavus* and *A. parasiticus* were found to be non-aflatoxigenic. The toxigenic properties of the aflatoxin-producing isolates of *A. flavus* and *A. parasiticus* were also studied and categorized by their fluorescence strength as shown in Table 3. Feed samples collected from the traditional Fulani dairy herds and institutional farms with low commercial activities showed higher occurrences (50.0% and 31.3%) of the aflatoxigenic strains of *A. flavus* and *A. parasiticus* respectively (Table 1).

Table 1: The occurrence and distribution of aflatoxigenic *A. flavus* and *A. parasiticus* along the different dairy settlements

Type of dairy settlement	Level of commercial activity	No. of feed samples tested	No. of samples +ve for *Aspergillus* spp	*A. flavus* + *A. parasiticus* +ve samples	*A. flavus* +ve samples	Aflatoxigenic +ve samples for	
						A. Flavus	*A. parasiticus*
Institutional	High	15	5	4	3	1	0
	Low	45	23	18	13	4	1
Commercial	High	15	7	5	3	1	0
	Low	15	9	6	5	1	0
Traditional Fulani dairy cooperatives	Mixed commercial activities	54	42	31	24	5	3
Total	-	144	86 (59.7%)	64 (44.4%)	48 (33.3%)	[a]12 (8.3%)	[b]4 (2.8%)

Table 2: The occurrence of *Aspergillus* spp in fresh and stored feeds among the different feed types

Feed type	Storage cond. of feed	N	Log_{10} CFU	% *A. flavus*	% *A. parasiticus*	% *A. fumigatus*	% *A. niger*	% *A. vesicolor*	% *A. tamarii*
Feed + concentrates	Fresh	24	4.1	42.0	8.0	14.0	24.0	11.0	1.0
	Stored	24	4.5	55.8	18.6	7.9	11.4	4.5	1.8
Feeds of grain origin	Fresh	24	3.2	44.0	10.0	0.0	29.0	17.0	0.0
	Stored	24	3.8	42.0	13.0	12.0	18.0	9.0	6.0
Dry pasture only	Fresh	24	3.4	76.0	0.0	0.0	22.0	2.0	0.0
	Stored	24	3.8	76.0	0.0	0.0	12.0	4.0	8.0
Total mean	-	-	3.8	55.9	8.3	5.7	19.4	7.9	2.8

Table 3: The distribution of *A. flavus* and *A. parasiticus* isolates by fluorescence characteristics

Aspergillus spp	Total No. of isolates (%)	No. of non-aflatoxigenic isolates (%)	No. of aflatoxigenic isolates (%)	Fluorescence strength of the aflatoxigenic isolates			Total
				+	++	+++	
A. flavus	48 (55.8)	36 (41.9)	12 (13.9)	3	8	1	12
A. parasiticus	16 (18.6)	12 (13.9)	4 (4.6)	0	4	0	4
Other *Aspergillus* spp	22 (25.6)	22 (25.6)	0	0	0	0	0
Total	86(100.0)	70 (81.4)	16 (18.6)	3	12	1	16

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Table 4: Quantitative determination of AFB1 amongst the fluorescence-positive isolates

S/No.	Isolate ID	Fluorescent strength of AF produced on NRDCA in 48 hrs	Quantitative Analysis of produced (AFB1) μgKg^{-1}	Mean concentration of AFB1 (μgKg^{-1}) produced by each fluorescence group
1	A	+++	22.45	22.45[a]
2	B	++	12.02	10.51[b]
3	C	++	14.97	
4	D	++	10.21	
5	E	++	13.10	
6	F	++	12.41	
7	G	++	12.01	
8	H	++	8.22	
9	I	++	11.80	
10	J	++	12.36	
11	K	++	10.24	
12	L	++	9.01	
13	M	++	11.81	
14	N	+	3.42	3.41[c]
15	O	+	4.61	
16	P	+	2.19	

A significant difference ($P<0.05$) exists between a, b and c

c) *PCR-based identification of fungal isolates from dairy cattle feeds*

All the suspected fungi based on the conventional microbiological methods represented by letters A-Z, yielded the expected IGS amplicon sizes of 674 bp as shown in Plate I. However, the negative control samples, the *E. coli* standard organisms EC1 and EC2 showed no amplicons (Plate 1).

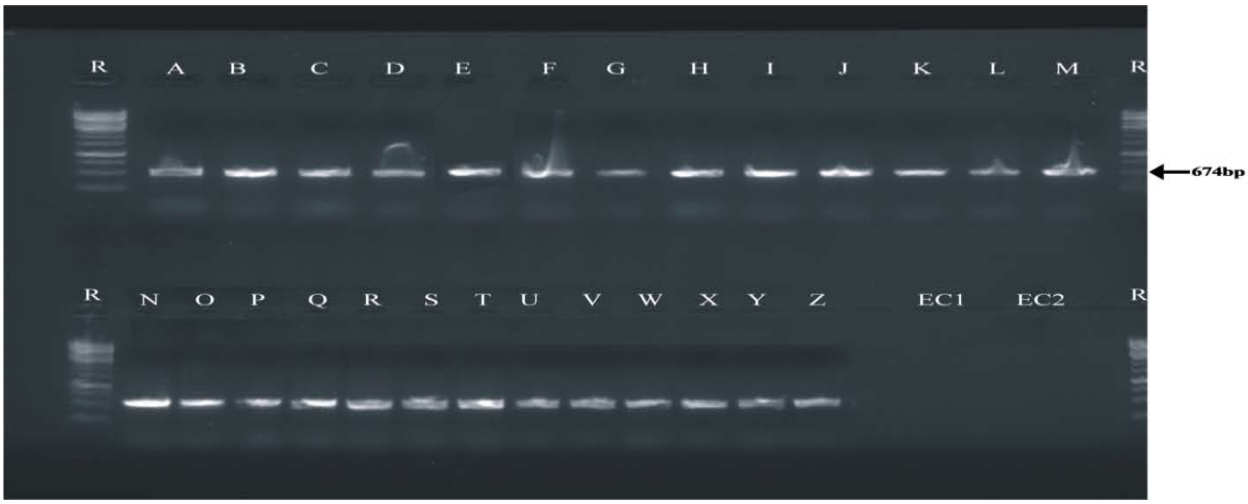


Plate I: Agarose gel electrophoresis of PCR product of IGS homologous to Fungi. Lane R (Pharmacia 1000 bp ladder), Lanes: A ('very strong' aflatoxigenic strain), B to M ('strong' aflatoxigenic strains), N to P (weak aflatoxigenic strains), Q to Z (atoxigenic strains) and EC1 and EC2 (strains of *E. coli* for –ve controls).

d) *PCR-based identification of Aspergillus spp among the identified fungal isolates*

The result of the primer set, *Asp1,* and *Asp2,* designed from V7 and V9 regions of *18S rRNA* confirmed and identified all the 86 isolates as *Aspergillus* species with the expected amplicon size of 500 bp as presented in Plate II. All the 16 UV-detected aflatoxigenic strains of Aspergillus yielded the expected amplicons (Plate II: A, B to M and N-P). Both *Fusarium* sp and *Rhizopus* sp used as standard and negative control organisms showed no amplicons (Plate II).

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Plate II: Agarose gel electrophoresis of PCR product of *18S rRNA* gene. Lane R (Pharmacia 1000 bp ladder), Lanes: A-P (represent *Aspergillus* spp) and Rz and Fs (represent standard *Rhizopus* sp and *Fusarium* sp respectively as –ve controls)

e) *PCR-based Detection and Identification of Aspergillus flavus strains that encode the specific gene, Fla*

The PCR-based detection and identification clearly showed a distinction between *A. flavus* and *A. parasiticus*. All the *A. flavus* yielded the expected amplicon size of 500 bp (Plate III). Among the 16 aflatoxigenic strains of *Aspergillus* section *Flavi* detected in this study, 4(25%) were negative for *A. flavus* specific amplicons as shown in Plate III. Partial sequencing of the amplified *IGS* regions successfully identified common strains of *A. flavus* in dairy feeds. The most common strain identified was *A. flavus* EGY1. Other less common strains identified include: *A. flavus* ITD-G11, *A. flavus* MJ49, A. *flavus* HKF30, *A. flavus* HKF13, *A. flavus* HKF49 and *A. flavus* 1985.



Plate III: Agarose gel electrophoresis of *fla* gene amplicon. Lane R (molecular size markers (Pharmacia 3000 bp ladder) Lanes E, F and K, L represent *A. parasiticus;* while other lanes represent *A. flavus*

f) *Restriction fragment analysis (PCR-RFLP) of A. flavus and A. parasiticus*

Further to the above molecular based differentiation between *A. flavus* and *A. parasiticus,* an enzyme based restriction analysis was carried out to differentiate the two species. Findings from agarose electrophoresis showed that in *A. flavus* strains, the PCR product was digested into three fragments of amplicon sizes 102bp, 210bp and 362bp (Plate IV). *A. parasiticus* showed one restriction site with two fragments of amplicon sizes 311bp and 362bp (Plate IV).

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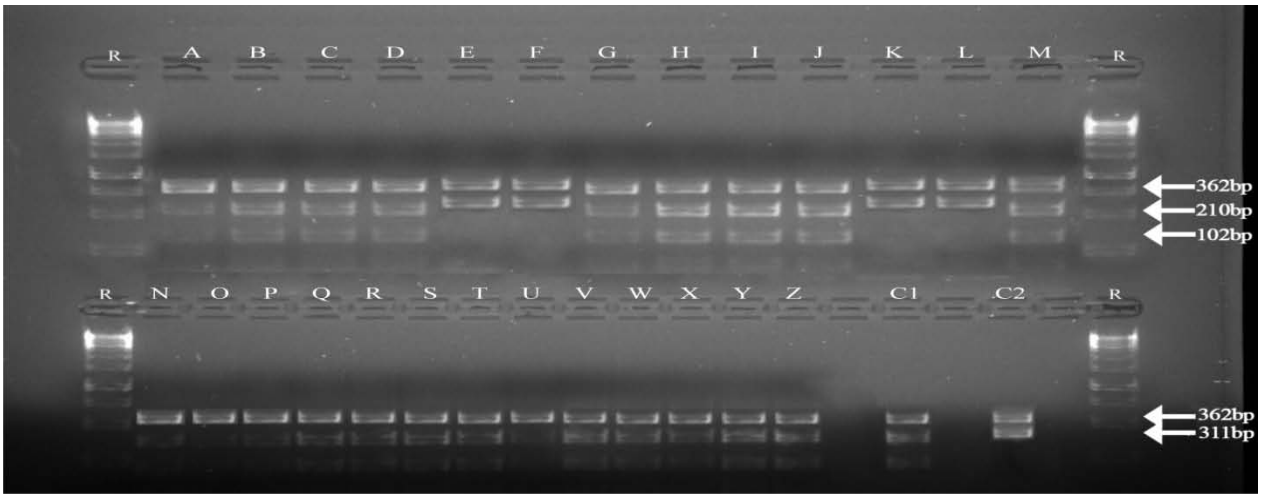


Plate IV: Agarose gel electrophoresis of *Bg III* digested PCR products of the *ITS* region of *A. flavus*. Lane R (Pharmacia 1000 bp ladder), Lanes E, F and K, L represent *A. parasiticus* while other lanes represent *A. flavus*. C1 and C2 (represent standard organisms of *A. flavus* and *A. parasiticus* used as positive controls)

g) Genes that encode aflatoxin biosynthesis

From the *in-vitro* study on the toxigenicity profile carried out to examine genes that encode aflatoxin biosynthesis, one particular isolate (A) presented a pronounced fluorescence. This isolate was earlier found to display a mono amplicon pattern for *O-methyl transferase* gene instead of the usual quadruplet amplicons (Plate VI), some other 12 strains (lanes B-M) showed strong fluorescence characteristics and three others (lanes N-P) showed weak fluorescence characteristics as summarily presented in Tables 3, 4. The non-aflatoxigenic strains had different amplicon patterns with few showing incomplete quadruplet amplicons for *aflR, omt, ver and nor* genes as presented accordingly in Plates V, VI, VII and VIII.

h) Determined level of AFB1 during the active growth on NRDCA

Concentrations of AFB1 produced during the active growth phase of the aflatoxigenic strains of *A. flavus,* and *A. parasiticus* as determined by the HPLC method are shown in Table 4. The analysis by the HPLC method confirmed the various degrees of fluorescence produced under a long wavelength UV. Mean concentrations of the different fluorescence groups showed a significant difference ($p < 0.05$) between them (Table 4).

IV. Discussion

Isolation and proper identification of aflatoxigenic strains of *A. flavus* require characterization and differentiation between aflatoxigenic and non-aflatoxigenic strains of the organism. Such differentiation was achieved in this study by demonstrating a link between produced aflatoxin on coconut-based agar medium and the presence of the corresponding aflatoxin biosynthetic genes. The strain differentiation of the organism in the present study is partly in agreement with the work of Latha *et al*. (2008) who differentiated aflatoxin-producing strains of *A. flavus* from the non-producing strains using *Aspergillus* Differential Media (ADM). Part of the current finding, however adopts the use of a locally prepared coconut agar medium in determining aflatoxin production under a long wavelength UV light (365 nm) in line with the report of Atanda *et al*. (2011).

The occurrence of *A. flavus* was observed to be higher in dairy cattle feeds fortified with concentrates than in any other feed types. This finding may be of relevance in the management of feed composition and preservation. Improved feeding is recommended for increased and quality yield; this depends on fortification of feeds with improved concentrates. Accensi *et al*. (2004) in a separate study also showed that *A. flavus* was the predominant species amongst the *Aspergillus* section *Flavi* isolated from mixed feeds. It, therefore, implies that a quality source of feed coupled with adequate preservation are fundamental requirements for achieving safety in animal production particularly dairy. The mean total count of *Aspergillus* species in this study was however found to be below the maximum limit recommended (5.0 $\log_{10}$ CFU/g of feed) for poor feed quality (Udom *et al*., 2012). Thus, comparing the current findings of 4.5 $\log_{10}$ CFU/g of feed with the previous works of 4.1 $\log_{10}$ CFU/g of feeds (Accensi *et al*., 2004; Udom *et al*, 2012), parts of which were conducted in Nigeria, may arouse safety questions about the quality of feeds fed to dairy cattle if contamination is unchecked. Such increasing trend in the level of fungal contamination as depicted by the rising Aspergillus colonial counts, portends serious public health concern.

The relatively low occurrence of aflatoxigenic strains of *Aspergillus* section *Flavi* (>11%) amidst high incidence rates of unclassified *Aspergillus* section *Flavi* (>59.0%) in the current study, may be tentatively

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considered as non-significant, but the instability and the genetic diversity shrouding the biosynthesis of aflatoxins may raise safety concerns. Udom *et al*. (2012) had, in one of his reports, articulated a correlation between the lower incidence of aflatoxigenic strains of *Aspergillus flavus* and the unpredictable high levels of AFB1.

About 50.0% of the total aflatoxigenic strains identified in this study was from the traditional Fulani dairy herds. It, therefore, implies public health risk, especially, in situation where many big institutional and commercial dairy farms depend on these traditional dairy herds for their production boosts. Such interrelationship between the conventional and the traditional dairy herds emphasizes the importance of the traditional dairies, thereby signifying the urgency of promulgating an act or strengthening the existing ones, if any, for effective management of both traditional and conventional dairy industries.

In the current study, the concurrent application of conventional microbiological and molecular methods identified a potential aflatoxin-producing (PAP) strains. The identification of the PAP strains is considered an important public health issue. Some genes have been found to play complementary roles in aflatoxin biosynthesis. In this study, *A. flavus* isolates were examined for the presence of the full amplicons of the quadruplet genes. The genes consist of 3 structural genes, *omt, ver,* and *nor* and one aflatoxin regulatory gene, *aflR,* involved in the biosynthesis of the aflatoxins, which also suggest full aflatoxigenic potential. Findings in this study showed that all the 16 isolates of the *A. flavus* and *A. parasiticus* identified as aflatoxin-producers by the stream of UV light, yielded a complete quadruplet amplicons. This PCR-based method of identification of the genes that encode aflatoxin production further stressed the capability and reliance of the microbiological method used for the preliminary aflatoxigenicity screening. Also, a quadruplet amplicon pattern was found amongst some of the observed non-aflatoxin producing strains detected during UV examination. This finding further elucidates the intrinsic and inherent aflatoxin-producing potential in the seeming non-producer of aflatoxins, should the conventional microbiological methods were used alone. The finding has also improved on the previous understanding that quadruplet amplicons are mostly associated with potential aflatoxin-producing strains. Genetic modification could occur in some of the strains during their growth phase on culture media. This genetic diversity may modify or even hamper the biosynthesis of aflatoxins among the potential aflatoxin-producing strains. This explanation is in agreement with the work of Abarca *et al*. (1988) which reported that certain instability of aflatoxin production may occur in aflatoxigenic strains growing on culture media. Such instability might be as a result of simple genomic drift as seen in the case of substitution of bases, leading to the formation of non-functional products (Criseo *et al*., 2001; Latha *et al*., 2008).

Findings in the present study also showed that all the aflatoxigenic and non-aflatoxigenic strains of *Aspergillus flavus* group examined possessed aflatoxin regulatory gene, except one isolate in the aflatoxigenic group. The isolate showed a distinct amplicon band for only *omt* gene. This strain, at the level of UV identification, displayed a very strong fluorescence (perhaps the strongest) characteristic under a long wavelength UV light (365 nm) indicating an apparently high level of aflatoxin production. Further confirmation showed that the amount of aflatoxin B1 produced by the strain was quite higher when compared with the other strains. One of the possible explanations is the omission of a particular gene, the aflatoxin regulatory gene, *aflR,* which may explain the strongest fluorescence characteristic associated with the strain, since *aflR* regulates the activities of the other structural genes. This finding may suggest the possible roles of gene alleles or alternative genes, not detected by the PCR method used. In previous studies, the *aflR* gene was shown to play a role in aflatoxin biosynthesis pathway by regulating the activities of the structural genes such as *omt, ver* and *nor* (Chang *et al*., 1992; Liu and Chu, 1998). Criseo *et al*. (2001) and Latha *et al*. (2008) had demonstrated the significant interdependent roles played between *omt* (a structural gene) and *aflR* (a regulatory gene) in biosynthesis of aflatoxins. Liu and Chu (1998) also demonstrated, through a hybridization technique that, *AFLR*, a product of *aflR* gene, regulates the expression of *omt*, a structural gene in the aflatoxin biosynthetic pathway.

Over expression of the structural gene, *omt,* may be caused by a state of relative inexpression of *AFLR.* This fact may be responsible for the 'very strong' fluorescence seen during the conventional examination of one of the aflatoxigenic isolates. Aflatoxin production is controlled by a mechanism of regulation of structural gene transcription in which the *aflR* plays a role. Liu and Chu (1998) also demonstrated the interdependent role of *AFLR,* a product of *aflR* and *Omt* genes in the final steps of aflatoxin biosynthesis. This may explain the inexpression of one or other structural genes amongst the non-aflatoxigenic strains isolated in the current study.

The instability associated with the aflatoxigenic *A. flavus* and *A. parasiticus* so far established in the preceding findings, has raised safety concerns about the use of non-producers of aflatoxins in the biological control of mycotoxin contamination of crops. The works of Dorner *et al*. (1999), Dorner and Cole (2002) and Abass *et al*. (2006) had advocated for the use of non-toxigenic strains of *Aspergillus flavus* for biological control of mycotoxin contamination of crops. The observed instability in the capacity of aflatoxin production among the different strains of *Aspergillus*

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section *Flavi* may be dependent on both climatic and environmental conditions. This aspect of the discussion may further explain the previous reports (Criseo *et al*., 2001; Latha *et al*., 2008).

The study identified the genus *Aspergillus* to species level. Two different species of *Aspergillus, A. flavus* and *A. parasiticus* were identified with *A. flavus* predominantly found to contaminate feed substances. This finding agrees with that of White *et al*. (1990), Gonzalez-Salgado (2008) and Latha *et al*. (2008) which demonstrated the use of primers in targeting DNA regions, *18S rRNA* and *Fla,* to identify the genus *Aspergillus* and *A. flavus* with the expected amplicon size of 674bp. A restriction fragment analysis of the PCR product of *aflR-aflJ* intergenic region using restriction endonuclease; *Bg III,* further affirmed the specificity of *Fla* gene in differentiating *A. flavus* from *A. parasiticus.* The 2 restriction sites at which the PCR product was cleaved into three and two fragments of 362, 210 and 102 bp and 362 and 311 bp confirmed *A. flavus* and *A. parasiticus* respectively which are in agreement with the findings of Somashekar *et al*. (2004).

This strain diversity found in the study agrees with the reports of early workers (Dorner *et al*., 1999; Abass *et al*., 2006) who also implicated many diverse strains of *A. flavus* in aflatoxin production. Successful aflatoxin control programs in any country may need a perfect understanding of strain specifics as regards aflatoxin production. The diverse toxigenic strains identified from cattle feed in this study may be a reflection of poor management of international trade amongst the neighboring African nations, thereby posing a wide range of public health risks to the communities concerned. Sequenced intergenic spacer region failed to differentiate between the strains of *A. flavus* and *A. parasiticus.* The close genomic similarity that exists between the two species may be responsible for this. Expanded whole genomic sequencing may, therefore be a better option towards resolving this complexity.

It was concluded that the occurrence of-*Aspergillus flavus* in dairy cattle feeds was high; the aflatoxigenic strains of *A. flavus* was however relatively low. Traditional dairies constitute the major public health risks as majority of the identified aflatoxigenic strains were associated with them. A potential aflatoxin-producing (PAP) strains and the role of aflatoxin regulatory gene on aflatoxin biosynthesis were established in the current study. This aspect of the work may be explored to biotechnologically raise strains of *A. flavus* with a modulated *aflR* function for biological control of aflatoxin biosynthesis in feed marketed for animal production in Nigeria and the world over.

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