Cattle Feed Contamination by Aspergillus Flavus and A. Parasiticus in both Conventional and Traditional Dairies

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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

Index terms—

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.

1 Introduction

flatoxins 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 o C and a suitable substrate (Tvrtkovi?, 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.

2 II.

3 Materials and Methods

4 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 A 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.

5 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 o 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).

6 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 nonfluorescence (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 ?l of the supernatant was diluted with 700 ?l of phosphate buffer, and the resultant solution used for the determination of AFB1.

7 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 NaCI, 1.2 g Na 2 HPO 4 , 0.2 g KH 2 PO 4 , 0.2g KCl, dissolved in 990 ml purified water) and pH adjusted to 7.0 with HCI. 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.

8 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. 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.

9 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.

10 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 º C until samples were retrieved.

11 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 **??**Ehrlich et al., , 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 ?l containing 15 units of enzyme, 4 ?l of buffer, 15 ?l of PCR product, and Ultrapure water up to 40 ?l. The reaction mixture was incubated at 37 o 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.

12 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 aflatoxinproducing capabilities among the isolated Aspergillus flavus. These include: Norsolorinic reductase (nor), Omethyl transferase (omt), Vesicolorin dehydrogenase (ver) and Aflatoxin regulated gene (aflR). The Primers used have been previously described (Geisen, 1996

13 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 crosscontaminate 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).

14 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.

15 III.

16 Results

17 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%

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).

18 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).

19 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).

20 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).

21 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.

22 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).

23 IV.

24 Discussion

Isolation and proper identification of aflatoxigenic strains of A. flavus require characterization and differentiation between aflatoxigenic and nonaflatoxigenic 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 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(D D D D)

G 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 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. G 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; **??**bass 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 aflatoxinproducing (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. [1]

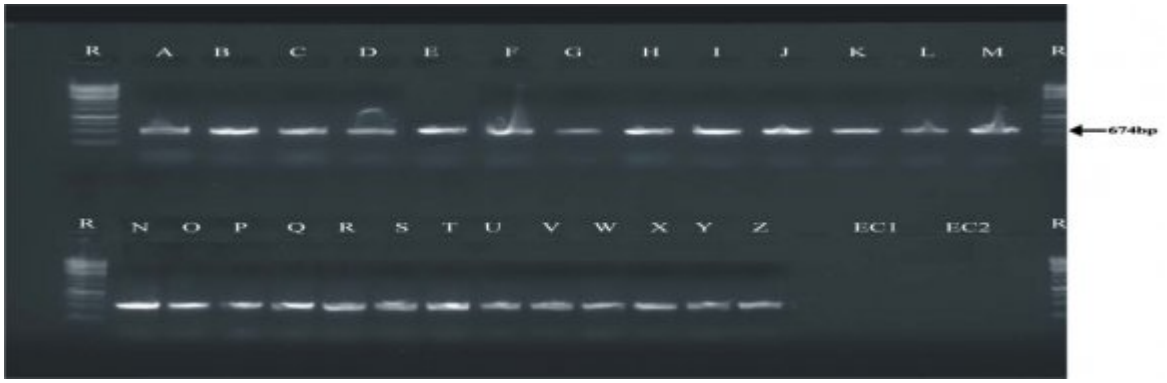

Figure 1: Aflatoxin

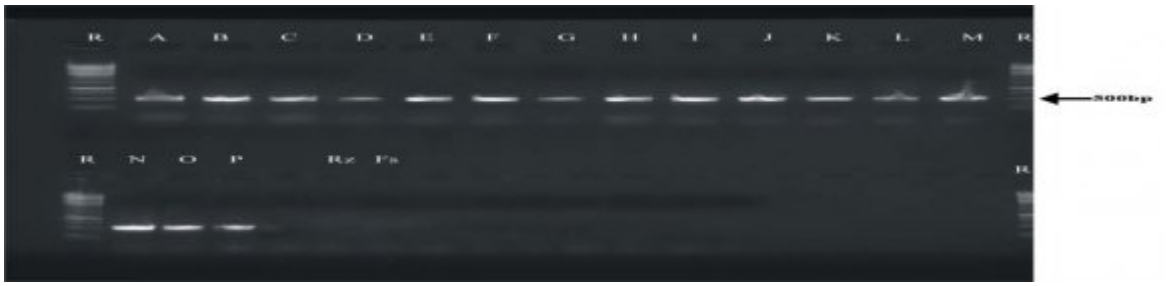

Figure 2:

Figure 3:

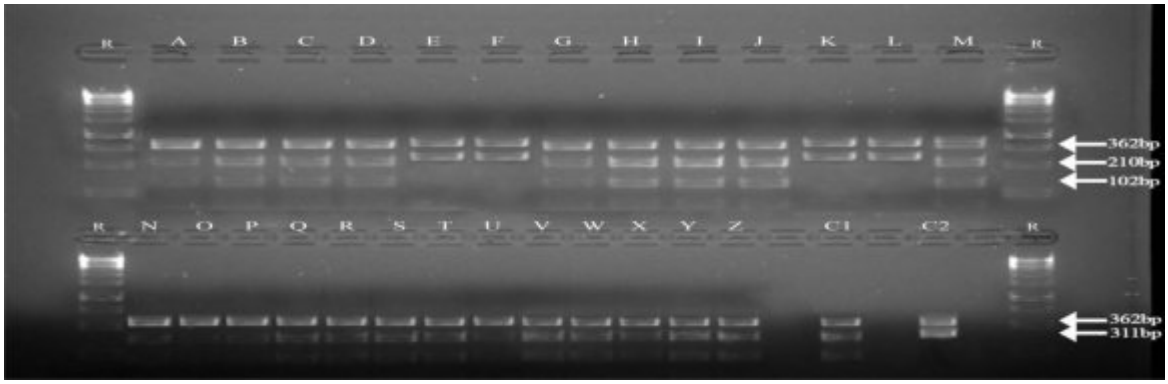

Figure 4:

1

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	
Institutional	High Low	15 45	5 23	4 18	3 13	1 4	0 1
Commercial	High Low	15 15	7 9	5 6	3 5	1 1	0 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%)

Figure 5: Table 1 :

2

Feed type	Storage cond. of feed	N	Log 10 CFU/g	% 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

Figure 6: Table 2 :

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

[Note: G]

Figure 7: Table 3 :

4

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	

[Note: A significant difference (P<0.05) exists between a, b and c]

Figure 8: Table 4 :

Figure 9:

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