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Real time PCR of Nor~1 (*aflD*) gene of aflatoxin producing fungi and its correlative quantization to aflatoxin levels in South African compound feeds

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ABSTRACT

Aflatoxins (AFs) are naturally occurring secondary metabolites. This toxin is principally produced by *Aspergillus flavus* and *Aspergillus parasiticus* in compound feeds worldwide. Compound feeds are feeds blended from various raw materials and additives. Contaminations of these feeds by AFs and its possible transmission into edible materials like milk, egg and organs of the body, are a serious problem. Expression of the Nor~1 (*aflD*) gene is the main factor responsible for AF production. For this reason, a study was carried out to establish a correlation between levels of AFs and determinant gene (Nor~1) in South African compound feeds. To achieve this, compound feeds (n = 30) were analyzed for *Nor~1* gene using real time polymerase chain reaction (RT-PCR), while AF levels in similar samples were estimated using high-performance liquid chromatography (HPLC) after an immune-affinity clean-up extraction procedure. Results indicated that AF levels in positive samples ranged from 0.7 to 33.0 ppb. These levels generally did not correlate ($R^2 = 0.093$) with those of Nor~1 gene in similar samples. Consequently, Nor~1 gene levels established via RT-PCR cannot be used as a predicting model for AFs in compound feeds. Only four of the feeds analyzed, specifically poultry feeds, contained levels of AFs above the regulatory limits of 10 ppb established in South Africa (S.A.). This should be considered unsafe when consumed on a continuous basis and may pose some health related problems especially when AFs are found together with other significant mycotoxins such as ochratoxins (OTs) and/or fumonisins (FBs).

1. Introduction

Aflatoxins which are principally produced by *Aspergillus flavus* and *Aspergillus parasiticus* represent a group of potent mycotoxins that contaminate compound feeds (worldwide, especially in the tropical and sub-tropical parts of the world). The consumption of feeds with significant amounts of AFs does not only affect animal health, but it also leads to serious economic losses due to poor animal husbandry arising from reduced feed intake/utilization, weight loss, poor immune function, decreased reproduction and even death in severe circumstances (Njobeh et al., 2012) resulting from Aflatoxicosis. Aflatoxicosis is a general term, referring to a disease condition in both animal and humans due to the exposure to AFs. A case in point was a recent outbreak of the disease in Gauteng Province of South Africa that killed over 220 dogs, with several others being affected after consuming pet food contaminated with high doses of AFs (Arnot et al., 2012). Several reasons can be attributed for this, but one of the most important is the fact that there is lack of proper management structures in place to limit AFs contamination with its implications on these feed commodities.

Production of AFs in these commodities takes place during the secondary metabolic activity of the aflatoxigenic fungi, involving the expression of Nor~1 (*aflD*) transcript gene (Yu et al., 2004; David, 2009) which is a determinant gene in the anabolic process of AFs. This gene encodes a reductase that converts norsolorinic acid to averantin. Subsequent reaction and enzyme conversions of averantin lead to aflatoxin synthesis (Dutton, 1988). This could either be Aflatoxin B_1 and Aflatoxin G_1 or Aflatoxin B_2 and Aflatoxin G_2, depending on the existing branch point. *Aspergillus* fungal genomic DNA analysis in naturally contaminated agricultural commodities can be performed via TaqMan fluorescent probe technology (María et al., 2008; Abdin et al., 2010). Sensitivity of the test demonstrates that the DNA amounts expressed which can be determined via RT-PCR chain reaction assay, account for the toxin production (Abdin et al., 2010). This technique has been reliable and absolute in the prediction of potential *Aspergillus* and aflatoxigenic risk in stored agricultural commodities. This molecular technique is suitable for rapid, automated and throughput analysis (Valsesia et al., 2005; Degola et al., 2007) in fungal identification. The principle behind this tool is based on the reactions with some specified primers that define

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a target sequence and an additional internal probe that hybridizes between these primers. As the gene responsible for AF production can be quantitatively detected in agricultural commodities, the attendant toxin can also be detected, quantified and correlated. Of these techniques, the principal immunochemical based assay is the commonly used enzyme linked immune-sorbent assay (ELISA). Other methods of detection and quantification of AFs are based on electrochemical and optical principles such as high performance liquid chromatography.

Although, studies have been carried out in agricultural products such as maize (Mayer et al., 2003), coffee, peanuts (Passone et al., 2010) and wheat (Sardinas et al., 2011) and the resultant correlations found and justified, no such studies have been carried out on compound feeds. The main objective of this present study therefore, was to correlate quantities of Nor~1 gene and AFs in compound feeds. Before establishing levels of AFs in compound feeds, we first of all isolated and estimated those AFs with the biosynthetic (Nor~1) gene by way of an application of diagnostic PCR procedures, in an attempt to establish if there was a correlation between the AFs and its relative gene.

2. Materials and methods

2.1. Materials

All reagents were of analytical or HPLC grade unless otherwise stated. Equipment included the Rotor Gene Amp 6000 detection system (Corbett Life Science — R080762, Australia); GeneAmp 5700R Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA); HPLC Spectra Physics SCM400 SYSTEM (Shimadzu Corporation, Kyoto, Japan) equipped with a LiChrospher 100 RP-18 column (250 mm × 4 mm i.d and 5 μm particle size) (Merck, Darmstadt, Germany), Waters Sentry™ guard column and a fluorescent detector (Shimadzu Corporation, Kyoto, Japan). The materials used included the aflaprep immunoaffinity columns (R-Biopharm AG; Darmstadt, Germany), standards of aflatoxins (AFs) i.e. aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin B_1 (AFB_1) and aflatoxin B_2 (AFB_2) (Sigma, Aldrich). Other material used included, nor probe and primers, micro Amp reaction tubes, TaqMan environmental Master Mix 2.0, QuantiFast pathogen PCR + IC kit, Qiagen (Whitehead Scientific), molecular grade water (nuclease free), SYBER green loading dye, plant and fungi DNA® extraction Mini and Maxi Kits (White Scientific Qiagen product, South Africa) and compound feed samples collected from South Africa.

2.2. Methodology

2.2.1. Fungal isolation and DNA extraction

Pure fungal isolates were harvested from feed samples according to Kaufman et al. (1968) and subsequently sub-cultured on yeast extract sucrose (YES) broth medium for 7 days. The extraction of DNA was performed using a DNA extraction Mini kit according to the manufacturer (Qiagen White Scientific). The purified DNA was stored at −20 °C until further analysis.

Extraction of DNA from a subset of 30 samples of the n = 92 samples of compound feeds presented in Table 2 was selected for DNA extraction and analysis by real time PCR. The extractions were performed using the DNeasy Plant Maxi Kit according to manufacturers' (Qiagen) instructions.

2.2.2. Real-time PCR analysis

A Rotor Gene Amp 6000 detection system (Corbett Life Science — R080762, Australia) was used to perform the real-time PCR cycle reactions. According to the published sequence of Trail et al. (1994), the primer/probe set used had the following nucleotide sequence: nortaq-1, 5′-GTCCAAGCAACAGGCCAAGT-3′; nortaq-2, 5′-TCGTGCATGTTGGTGATG GT-3′; nor probe, 5′-TGTCTTGATCGGCGCCCG-3′ enclosing an amplicon of 66 bp from nucleotide 782 to 847. FAM labeled Nor~1 probe (QuantiFast pathogen PCR + IC kit, Qiagen, Whitehead Scientific) was used for PCR as suggested by the manufacturer (Whitehead Scientific). Individual reactions contained 2.5 μl of the DNA sample solution which was mixed with 5 μl master mix (Taq DNA polymerase, dNTPs, MgCl2 and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR), 3.5 μl of the primers i.e. nortaq-1 (1.75 μl), nortaq-2 (1.75 μl) each, 0.5 μl probe (0.5 nM) and 13.5 μl nuclease free water to make up a reaction volume of 25 μl. The PCR was performed in Micro-Amp reaction tubes placed in a 36-well rack of the GeneAmp 5700R Sequence Detection RT-PCR System. Incubation proceeded for 2 min at 50 °C to allow for cleavage of uracil-Nglycosylase. AmpliTaq Gold activation was done by incubating for 10 min at 95 °C. The following temperature range i.e. 95 °C for 20 s, 55 °C for 20 s and 72 °C for 30 s were used for the 35 PCR cycles.

To provide accurate results of the RT-PCR quantification, a standard curve was generated. Standard curves were then analyzed and generated using reference DNA (Nor~1) of known concentration (2.82 ng) extracted from fungal mycelium, before analyzing those of unknown concentrations extracted from the feed samples. A serial 5 × 10 fold dilutions (10^{-1} to 10^{-6}) from aliquots of DNA of known concentration were prepared using the Taqman method of Selma et al. (2008), with gene specific primers and probes and the best dilution curve generated was chosen for further analysis.

The specificity of primers, probes and validity of the reaction system for the real time PCR was assayed to test for the genomic DNA in the samples and that of fungal species (Table 1) isolated in similar compound feeds. This was done in triplicate with a negative control template and positive control template DNA but with 2.5 μl of the internal control DNA (ICD) and internal control assay (ICA).

2.2.3. Aflatoxin analysis

Aflatoxin extraction from the feed samples using an immunoaffinity column (R-Biopharm Rhone Ltd) was achieved following an extraction and clean-up protocol described by Candlish et al. (1998) with modifications using the version PO7/V15/26.01.05 aflaprep kit. The milled sample (12.5 g) and 1 gram NaCl were weighed into a solvent resistant blender jar into which 62.5 ml methanol and distilled water (60:40, v/v) were added and blended for 60 s. The extract was filtered and diluted with distilled H_2O (62.5 ml) which was mixed thoroughly by swirling. Sample extract (25 ml) was passed through a filter paper (Whatman No. 4) and 10 ml of the filtrate obtained (equivalent to 1 g of sample) was passed through an immuno-affinity column at a flow rate of 2–3 ml/min, after which, the immuno-affinity column was washed using 10 ml of phosphate buffered saline (PBS) at a flow rate of 5 ml/min. The analytes were then eluted (1 drop/s) using 1 ml of methanol and collected in an amber vial. Back flushing was employed thrice with the eluent to ensure complete release of AFs into the solution. The extract was dried in a fume cupboard using N_2 gas and stored at 0 °C until use for further analysis.

Sample extracts were analyzed for AFs by fluorescence detection following the HPLC method described by Ahsan et al. (2010) with some modifications. The HPLC system used for this assay was a Shimadzu Corporation (Kyoto, Japan) LC-20AB liquid chromatography equipped with CBM-20A communication bus module, LC-20AB degasser, CTO-20A column oven, SIL-20A auto sampler, RF-10AxL fluorescence detector,

Table 1
Specificity of reactions as shown with DNA of different fungi strains.

Fungi strains	RT PCR reaction with Nor~1 primers & probe
Aspergillus flavus	+
Aspergillus parasiticus	+
Aspergillus ochraceus	−
Aspergillus niger	−
Fusarium verticillioides	−
Fusarium proliferatum	−
Penicillium citrinum	−

+ Ct value of 35 cycles.
− Ct values above 35 cycle (39 to 40).

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Kobra cell RID-10A refractive index detector and SPD-M20A photodiode array detector linked to an LC solutions module (Software Release 1.22). Extracts were re-dissolved in 1 ml CH_3OH, filtered through a 0.2 μm Millipore filter and filtrate used as analyte solution. Chromatographic separation of analytes and standards was performed by passing the analyte or standard through a Waters Sentry™ guard column (Waters, Milford, USA) and a Symmetry column (250 × 4.6 mm i.d., 5 μm particle size). Aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) were detected without derivatization by using a fluorescent detector. The analysis was performed isocratically at a column temperature of 30 °C with excitation and emission wavelengths of 365 and 440 nm, respectively. The mobile phase, pumped at a flow rate of 1.2 ml/min, was water/acetonitrile/methanol (55:22.5:22.5, (v/v/v)). The injection volume of the analyte and standard toxins used was 20 μl. Calibration curves were obtained from increasing concentrations of standards, i.e., AFB_1 (2.5, 5 & 10 ppb), AFB_2 (0.5, 1 & 2 ppm), AFG_1 (2, 4 & 8 ppm) and AFG_2 (1, 2 & 4 ppm). The peak areas and retention times of AFs in sample extracts were used to determine their concentrations per sample, using the mathematical formula below, with respect to the limit of detection and limit of quantification.

$$\text{Sample AF (ppb)} = [\text{Sample peak area} \times \text{standard (ppb of AF)}]/\text{Standard peak area}$$

Recoveries of AFs were determined by spiking 25 g of feed sample containing no detectable amount of AFs with known amounts of AFB_1, AFB2, AFG_1 and AFG_2 standards. The spiked samples were thoroughly mixed and incubated at room temperature in a fume cupboard for at least 1 h before further analysis. Samples were prepared in triplicates and extraction for AFs carried out as previously described.

2.2.4. Statistical analysis

Dilutions were analyzed in duplicate on a 36-well plate Rotor Gene Amp 6000 detection System and standard curves generated by the RG-6000 system software (Rotor-Gene 1.7.94). Linear correlation analysis using SigmaStat 3.5 for Windows (Systat Inc., 2006) was done to establish the coefficient of linear correlation at 95% intervals for AF concentrations and DNA concentration. The slope of the y-intercept and R^2 values was noted and efficiency was determined from the slope of the regression line. The slope of the y-intercept and R^2 values was noted and used to calculate the slope of the curve (y) and correlation coefficients (R^2) in order to estimate the efficiency of the PCR assays.

3. Results

3.1. Real time-PCR data

Results obtained from the RT-PCR analysis indicated the sensitivity, specificity and validity of the PCR primers/probe experimental reaction setup. The spacing between each amplification curve based on the serial dilutions was approximately 3.36 cycles. This generated a standard curve (Fig. 1) with a slope of approximately −3.361. Template degradation was noted and quantification sensitivity did not decrease since the y-intercept of 32.9 was not too.

A clear relationship between initial DNA concentration and changes in fluorescence showed an amplification curve with known template concentrations. DNA isolated from the fungi suspension in a media broth, in a range of 2.5×10^3 to 2.5×10^7 CFU/g reaction showed progressively lower threshold (C_t) values. Accordingly, with the observed data, mean percentage recovery for DNA isolated was 94.7 ± 16.4% (n = 3). Data indicated an inverse linear correlation between DNA/pg and Ct with slope (mean = −2.8) and r values similar in three independent assays (mean = 0.99), indicating the high linearity of RT-PCR system. With these results, the primer/probe system used (nortaq-1, nortaq-2, norprobe) appeared sensitive and accurate for detection of the Nor~1 fragments extracted from the compound feed samples.

Real-time PCR was performed for the determination of reaction specificity, using different species of environmental fungi strains isolated from feeds. Primers and probes used in the reaction system showed high specificity (Table 1) as expected for aflatoxigenic fungi characterized using Nor~1 gene and a negative result was found for other fungi that did not contain the Nor~1 gene.

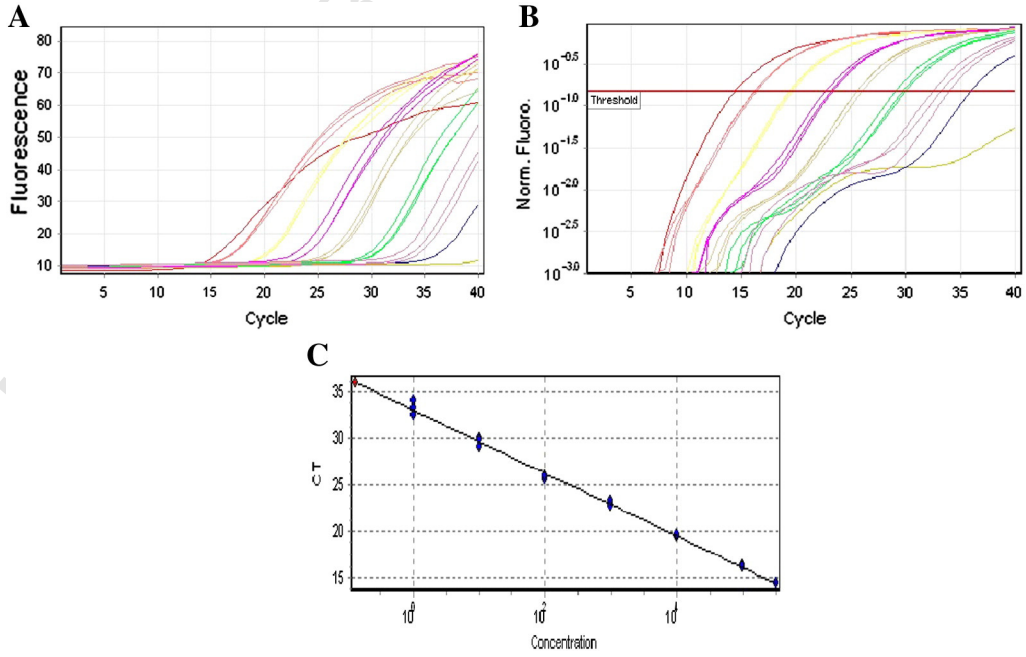

Fig. 1. Amplification plot of real-time PCR reaction targeted against the Nor~1 gene with different dilutions of the 66-bp Nor~1 fragment (— 10^6, ……. 10^5,……. 10^4,……10^3,……. 10^2, …….. 10^1). Fluorescence emission plotted against the number of PCR cycles and threshold value was set at 10^{-10}. Efficiency = 0.987. A: raw data cycling A (Green); B: quantitation data cycling A; and C: corresponding quantitation standard curve generated on real-time PCR analysis. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

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3.2. High performance liquid chromatography detection and quantification

The experimental calibration curves used in AFs quantifications were obtained with known concentrations of standards and correlation coefficient values of (R^2) of ≥0.9993 were obtained.

The limit of detection (LOD) of AFs was higher for HPLC as more samples (73%) were positive for AFs than for TLC (55%) with AF recoveries as follows: AFB_1 in samples was recovered at a rate (v/v) of 98.7 ± 1.0% (mean ± SD); AFB_2 at 99.5 ± 0.8%, AFG_1 at 98.1 ± 0.7% and AFG_2 at 96.9 ± 0.9%.

Data on the incidence and levels of AFs contamination (not adjusted based on recovery) are summarized in Table 2. Overall, poultry feeds were the most contaminated feeds with AFs being recovered in 62% of samples analyzed when compared to cattle (57%), white pig (5%) and horse (3%) feeds were the least contaminated substrates. The highest contamination levels of AFs were found in poultry feeds ranging from 0.005–77 ppb (mean: 21.7 ppb), while much lower levels were estimated in cattle feeds (mean: 9.1 ppb; range: 0.007 to 18.27 ppb), with horse and pig feeds having the least mean contamination levels that varied between 0.005 and 0.25 ppb (max. 0.7 ppb).

3.3. Correlation of aflatoxin level to Nor~1 gene

The incidence of AFs recovered livestock feed samples was 78.4% based on HPLC results. However, the concentration of DNA varied ($P > 0.05$) amongst the feeds (Table 3). This variation was also a characteristic feature of the Nor~1 gene detected and quantified in the sample feeds. The picogram (pg) DNA/mg feed was also correlated with AFs measured using HPLC. Correlation coefficient (r) determined for the target DNA gene and AF levels in the feed types had a range between −0.021 to 0.70 for the different feed samples (Table 3) based on picogram DNA/mg feed. Linear correlation analysis using SigmaStat 3.5 for Window (Systat Inc., 2006) was done to establish the coefficient of linear correlation which was 0.795 at 95% interval for the AF concentration and DNA concentration.

4. Discussion

Real time-PCR has been employed in the analysis of some commodities. Bagnara et al. (2000) determined copy numbers of this Nor~1 gene, which were poorly correlated. The study of Passone et al. (2010), established a correlation between Nor~1 gene expression and CFUs of these *Aspergillus* species ($R^2 = 0.613$, $P < 0.0001$) in naturally stored agricultural products. However, that particular study was not

Table 3
Aflatoxin (AF) load in correlation to Nor~1 gene concentration in animal feeds from South Africa.

Feed type	AF (ppb)	*Nor~1* gene (pg DNA/mg feed)
Poultry feeds (n = 15)		
Broiler (n = 6)		
EA 003	5.0	19.3 ± 1.1
EA 034	30.0	5.3 ± 3.2
EA 040	1.8	0.2 ± 0.1
EA 055	33.0	45.7 ± 3.02
EA 087	17.3	150 ± 27.9
EA 088	72.0	24.8 ± 1.0
Layer (n = 5)		
EA 001	1.2	25.1 ± 18.2
EA 011	3.6	7.6 ± 6.1
EA 022	7.0	110.1 ± 5.1
EA 030	8.6	159.2 ± 3.8
EA 089	4.8	20.9 ± 1.4
Breeder (n = 4)		
EA 010	4.8	0.9 ± 0.3
EA 055	5.5	2.6 ± 6.5
EA 060	7.2	2.7 ± 0.9
EA 090	7.4	8.4 ± 2.6
Cattle feeds (n = 11)		
Dairy (n = 5)		
EA 064	3.1	3.9 ± 1.4
EA 066	1.9	11.3 ± 1.0
EA 068	1.7	4.8 ± 1.2
EA 070	3.1	8.7 ± 5.6
EA 091	4.5	17.5 ± 2.9
Calf grower (n = 4)		
EA 072	2.4	0.1 ± 0.0
EA 074	2.2	20.6 ± 4.5
EA 076	4.5	4.5 ± 2.8
EA 078	2.6	2.5 ± 0.9
Finisher (n = 2)		
EA 079	2.3	5.9 ± 3.0
EA 083	2.0	0.3 ± 0.2
Other feeds (n = 5)		
Horse (n = 3)		
EA 013	nd	nd
EA 085	1.0	2.3 ± 0.9
EA 086	nd	5.1 ± 2.5
Pig (n = 2)		
EA 014	nd	2.0 ± 1.4
EA 017	0.7	0.6 ± 2.4

S.D: Standard deviation, nd: not detected.
DNA (pg/mg) was average of three replicates and mean ± SD. n: number of samples analyzed is 30.
Overall $R^2 = -0.093$ refers to the correlation between aflatoxin measured in the samples using HPLC (the 'ppb' column) and real time PCR (the 'Nor~1' gene column).

Table 2
Estimates (ppb) of aflatoxins in animal feeds from South Africa obtained by HPLC.

		AFB_1			AFG_1			AFB_2			AFG_2			
Feed	N[a]	N[b]	Range[c]	Mean[d]	N[b]	Range[c]	Mean[d]	N[b]	Range[c]	Mean[d]	N[b]	Range[c]	Mean[d]	TN[e]
Poultry														
Broiler	28	20	1.2–72.0	21.7 ± 0.5	17	3.8–48.2	16.8 ± 0.1	8	1.5–10.1	3.7 ± 0.2	1	–	–	46
Layer	20	13	3.6–8.6	2.2 ± 0.7	12	3.3–5.1	2.8 ± 0.3	8	0.01–3.0	1.08 ± 0.5	1	–	–	35
Breeder	14	9	3.0–7.4	1.8 ± 0.2	7	0.5–5.1	1.3 ± 0.5	5	2.8–3.3	1.1 ± 0.3	–	–	–	21
Cattle														
Dairy	11	8	0.8–4.5	1.0 ± 0.5	7	3.0–3.5	3.1 ± 0.3	7	0.2–1.1	2.6 ± 0.5	–	–	–	22
Calf	8	5	0.5–4.5	1.1 ± 0.8	5	0.7–4.9	1.5 ± 0.2	3	0.2–1.0	1.3 ± 0.4	–	–	–	13
Finisher	6	5	0.2–2.3	0.7 ± 0.6	4	0.5–3.1	1.0 ± 0.6	4	0.1–1.8	0.6 ± 0.4	–	–	–	13
Others														
Horse	3	1	–	0.7 ± 0.4	0	–	–	0	–	–	–	–	–	01
Pig	2	1	–	0.5 ± 0.3	0	–	–	0	–	–	–	–	–	01
Total	92													152

[a] Number of samples analyzed.
[b] Number of samples positive with AF.
[c] Range levels of AF contents for positive sample.
[d] Mean levels of AF contents for positive sample.
[e] Number of total samples positive with AFs.

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based on aflatoxin load, but on CFUs. Recent study of Rodríguez et al. (2012) and the approach of Babu and Murianaa (2011), presented a model system that could easily be adapted for aflatoxin detection in a variety of food and feed samples but not with respect to Nor~1 gene.

This present study is the first to not only identify AF producing fungi i.e. *A. flavus* and *A. parasiticus*, but to also compare the influence of relative quantification of the Nor~1 gene in relation to AF load in livestock feeds. This was based on Nor~1 gene levels in correlation to that of estimated AF levels in similar feeds. This provided information that relates molecular characteristics to morphological parameters, which influence aflatoxin production by aflatoxigenic fungi. The Nor~1 gene, when compared to aflatoxin load, is a constant constitutive expression of the gene (Mayer et al., 2003) in the metabolic phases of the aflatoxigenic fungal species. Results in this study confirmed that there was no statistical correlation between aflatoxin load and the Nor~1 gene expression levels. The reasons for such an observation cannot be explained with any certainty. It may be that even though the Nor~1 gene was present in the feeds, it may or may not have been expressed to produce AFs.

It is possible that propagules (vegetative structure) of *A. flavus* and *A. parasiticus* contain the Nor~1 gene, but the presence of this gene may not lead to AF/s production. Schmidt-Heydt et al. (2007) also examined *Penicillium verrucosum* populations, ochratoxin A (OTA) and the OTA polyketide synthesis gene (*otapks*Pv) expression in feeds and showed a good correlation between the *otapks*Pv expression and OTA production, but this was paralleled by CFUs of *P. verrucosum*. In this present study, there were samples (3/30) that did not contain Nor~1, but were contaminated with AFs. This may indicate that there was no permissible detection because of incomplete induction of the gene and that the gene presence/expression may vary with respect to physiological conditions as viewed by Scherm et al. (2005). It could also be that some other genes i.e. *aflR* and *aflQ* present in some strains of *A. flavus* and *A. parasiticus* involved in the AF biosynthetic pathway (Scherm et al., 2005) or other genes present in other less common AF producing fungi (*Aspergillus normius, Aspergillus bombycis*, and *Aspergillus pseudotamarii*) may account for this observation.

The discrepancy of the Nor~1 gene in the compound feeds, in correlation to the differential AF extract concentrations, may be due to the presence of differences in the chemical compositions of the feeds, (adulterations of the toxin). This may be due to the fact that these feed samples were obtained from different feed manufacturers in South Africa and therefore have different formulations in their production formula/compositions. Nevertheless cases of AF contaminations abound, either at the pre or post production phases involved in feed manufacturing, having been regarded as "non avoidable contaminants". Consumption of these AF contaminated feeds by livestock even at their lowest levels of occurrence, if not continually monitored, regulated and/or checked, could cause a number of health related issues, which if not recognized and treated promptly may well lead to the animal's premature demise.

5. Conclusion

Real time PCR of the Nor~1 gene and the correlative quantitation to the AF concentrations in compound feeds from South Africa need to be studied further. The determination of Nor~1 gene expression in relation to AFs has been observed for example in other food commodities, making the production/load in livestock feeds by the real-time PCR system possible. As a model for livestock feed matrix, a real-time reverse transcription-PCR system to establish AFs in feeds is necessary, so as to completely characterize the mycobiota of all livestock feeds. It represents a useful tool for further study, in order to determine correlations between Nor~1 expression and aflatoxin load by all AF producers found in these feeds. Help in predicting the potential risk of AF production in these particular livestock feeds based on Nor~1 gene levels alone may be an initial step towards improving overall livestock feed safety.

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References

Abdin, M.Z., Ahmad, M.M., Javed, S., 2010. Advances in molecular detection of *Aspergillus*: an update. Arch. Microbiol. 192, 409–425.

Babu, D., Murianaa, P., 2011. Immunomagnetic bead-based recovery and real time quantitative PCR (RT iq-PCR) for sensitive quantification of aflatoxin B1. J. Microbiol. Methods 86, 88–194.

Bagnara, A., Mayer, Z., Geisen, R., 2000. Quantification of *Aspergiullus flavus* BFE96 by sybr green Q-PCR in black pepper. Mycotoxin Res. 16, 244–247.

Candlish, A.G., Haynes, C.A., Stimson, W.H., 1998. Detection and determination of aflatoxins using affinity chromatography. Int. J. Food Sci. Technol. 23, 479–485.

David, R.G., 2009. Functional and comparative genomics of *Aspergillus flavus* to characterize secondary metabolism. A Dissertation Submitted to the Graduate Faculty of North Carolina State University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy, Functional Genomics. (Raleigh, North Carolina).

Degola, F., Berni, E., Dall'asta, C., Spotti, E., Marchelli, R., Ferrero, I., Restivo, F., 2007. A multiplex RT-PCR approach to detect aflatoxigenic strains of *Aspergillus flavus*. J. Appl. Microbiol. 103, 409–417.

Dutton, M.F., 1988. Enzymes and aflatoxin biosynthesis. J. Microbiol. Rev. 52, 274–295.

María, V.S., Pedro, V.M., Rosa, A., 2008. Real-time PCR based procedures for detection and quantification of *Aspergillus carbonarius* in wine grapes. Int. J. Food Microbiol. 122, 126–134.

Mayer, Z., Bagnara, A., Farber, P., Geisen, R., 2003. Quantification of the copy number of nor-1, a gene of the aflatoxin biosynthetic pathway by real-time PCR, and its correlation to the cfu of *Aspergillus flavus* in foods. Int. J. Food Microbiol. 82, 143–151.

Passone, M.A., Rosso, L.C., Ciancio, A., Etcheverry, M., 2010. Detection and quantification of *Aspergillus* section *Flavi* spp. in stored peanuts by real-time PCR of nor-1 gene, and effects of storage conditions on aflatoxin production. Int. J. Food Microbiol. 138, 276–281.

Rodríguez, A., Rodríguez, M., Isabel Luque, M., Martín, A., Córdoba, J., 2012. Real-time PCR assays for detection and quantification of aflatoxin-producing molds in foods. Food Microbiol. 31, 88–89.

Scherm, B., Palomba, M., Serra, D., Marcello, A., Migheli, Q., 2005. Detection of transcripts of the aflatoxin genes aflD, aflO, and aflP by reverse transcription-polymerase chain reaction allows differentiation of aflatoxin-producing and non-producing isolates of *Aspergillus flavus* and *Aspergillus parasiticus*. Int. Food Microbiol. 98, 201–210.

Schmidt-heydt, M., Richter, W., Michulec, M., Butinger, G., Geisen, R., 2007. Molecular and chemical monitoring of growth and ochratoxin A biosynthesis of *Penicillium verrucosum* in wheat stored at different moisture conditions. Mycotoxin Res. 23, 138–146.

Selma, M.V., Martínez-culebras, P.V., Aznar, R., 2008. Real-time PCR based procedures for detection and quantification of *Aspergillus carbonarius* in wine grapes. Int. J. Food Microbiol. 122, 126–134.

Systat Inc., 2006. SigmaStat 3.5 for Windows. Systat Software, Inc., Point Richmond, CA, 94804–2028, USA.

Trail, F., Chang, P.K., Cary, J.W., Linz, J., 1994. Structural and functional analysis of the nor-1 gene involved in the biosynthesis of aflatoxins by *Aspergillus parasiticus*. Appl. Environ. Microbiol. 60, 4078–4085.

Valsesia, G., Gobbin, D., Patocchi, A., Vecchione, A., Pertot, I., Gessler, C., 2005. Development of a high-throughput method for quantification of *Plasmopara viticola* DNA in grapevine leaves by means of quantitative real-time polymerase chain reaction. J. Am. Phys. Soc. 95, 672–678.

Yu, J., Bhatnagar, D., Cleveland, T.E., 2004. Completed sequence of aflatoxin pathway gene cluster in *Aspergillus parasiticus*. J. Fed. Eur. Biochem. Soc. 564, 126–130.

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