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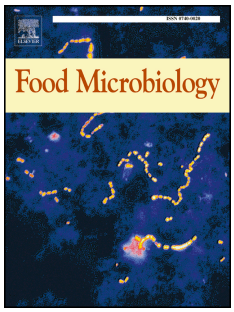

Morphological and molecular identification of filamentous *Aspergillus flavus* and *A. parasiticus* isolated from compound feeds in South Africa

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Morphological and molecular identification of filamentous *Aspergillus flavus* and *A. parasiticus* isolated from compound feeds in South Africa

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ABSTRACT

Isolation of filamentous species of two *Aspergillum* genera from compound feeds produced in South Africa, and subsequent extraction of their individual DNA in this study, presents a simple but rapid molecular procedure for high through-put analysis of the individual morphological forms. DNA was successfully isolated from the *Aspergillus* spp. from agar cultures by use of a commercial kit. Agarose gel electrophoresis fractionation of the fungi DNA, showed distinct bands. The DNA extracted by this procedure appears to be relatively pure with a ratio absorbance at 260 and 280 nm. However, the overall morphological and molecular data indicated that 67.5 and 51.1% of feed samples were found to be contaminated with *A. flavus* and *A. parasiticus,* respectively, with poultry feed having the highest contamination mean level of 5.7×105 CFU/g when compared to cattle (mean: 4.0 × 106 CFU/g), pig (mean: 2.7 x 104 CFU/g) and horse (1.0 x102 CFU) feed. This technique presents a readily achievable, easy to use method in the extraction of filamentous fungal DNA and it's identification. Hence serves as an important tool towards molecular study of these organisms for routine analysis check in monitoring and improving compound feed quality against fungal contamination.

Key words: Compound feed, *Aspergillus* fungi, morphological, molecular, DNA, electrophoresis

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1. INTRODUCTION

Fungi are continuous threat to livestock feeds of economic importance such as compound feeds. They may affect feed either directly by causing mechanical damage throughout feeding, or indirectly by secreting and spreading mycotoxins such as aflatoxins in the case of aflatoxin producing fungi. The common fungal genera contaminating compound feeds in South Africa are those belonging to the *Fusarium, Penicillium* and *Aspergillus* genera. The predominant *Aspergillus* species are *Aspergillus flavus* and *A. parasiticus* elaborating the deterioration of compound feeds to reduced health and performance of those animals fed on such feeds. They are ubiquitous in nature and for some time, have become an increasing cause of life-threatening opportunistic diseases (Linden *et al.,* 2003). These fungi proliferate in terms of growth and increased aflatoxin production, exhibiting high levels of disease pathogenicity in their diverse forms. This has resulted in the growing interest in molecular biology of these fungi warranting acceleration in genomic research. Accurate identification of fungal pathogen is in many cases, a prerequisite for effective management of the diseases they cause and for ecological or population genetics studies (Gherbawy and Voigt, 2010). However, these fungal species are much more similar to each other and accurate identification to species level could not be possible. Hence, it is paramount that their morphological and molecular characteristics with respect to DNA presences are investigated, using the methods of fungal isolation and screening making use of macro- and microscopic analysis, fungal DNA extraction, polymerase chain reaction (PCR) and an agarose gel electrophoresis. Current advances in biotechnology, molecular genetic marker have been employed for rapid identification of different species of fungi (Lieckfeld and Seifert, 2000, Attanayake *et al.* 2009). Nevertheless, isolation of intact DNA is critical for a number of molecular analyses such as cDNA production and transcriptional output quantitation (Selma *et al.,* 2008). Advancements towards identifying fungal species are by way of using DNA markers, developing DNA barcodes that are diagnostics of target species using species-oligonucleotides (Druzhinina *et al*., 2005). However, extraction processes of DNA from *Aspergillus* spp. depend on cell disruptions, nuclease inactivation and subsequently, the extraction of the molecule. A broad range of molecular manipulations of these fungi are now possible. These include gene disruption, PCR and Real time PCR (RT-PCR) applications as well as DNA-based epidemiological studies (Jin *et al.,* 2004). Each of these techniques requires the recovery of good-quality genomic DNA. Most DNA extraction protocols for *Aspergillus* spp. rely on mechanical isolation methods that employ grinding mycelia after freezing them in liquid nitrogen or glass bead disruption, followed by additional purification steps (Guglielmo *et al.,* 2008). These proceed after microbial growth of the fungi to harvesting colonies for DNA extraction. The morphological examination of these fungi, against its relative molecular technique with respect to AflD, indicates absolute relative high through-put in the identification of the two fungal species.

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2. MATERIALS AND METHODS

2.1 Sampling

Samples of compound feeds which include those for poultry, cattle, pig and horse were donated by different feed manufacturers in South Africa under the auspices of the South African Feed Manufacturers' Association (AFMA). Samples (about 500 g each) were collected following the standard sampling protocol of Candlish *et al*. (1998), to give a representative sample which were sealed in sampling plastic bags and taken to the laboratory of the Food, Environmental and Health Research Group, Faculty of Health Sciences, University of Johannesburg for analysis.

2.2 Fungal screening

A microbiological analytical procedure of Kaufman *et al*. (1968) with some modifications was used in this study and carried out under aseptic condition. Accordingly, 1 g of ground sample was weighed into a sterile test tube, suspended in 9 ml of sterile Ringer's solution and vortexed. The suspension (1 ml) was serially diluted in 9 ml of the Ringers' solution further to 10^{-6}. One ml from each dilution was cultured on Ohio Agricultural Station agar (OAESA) and potato dextrose agar (PDA) and incubated for 5-7 days at 30°C. After incubation, fungal colonies were counted macroscopically using a colony counter. Colony forming units per gram (CFU/g) of sample was calculated. Isolates of *A. flavus* and *A. parasiticus* were further sub-cultured on PDA, Czapek yeast agar (CYA) and malt extract agar (MEA) under aseptic conditions and incubated at 30°C for 7 days. Pure colonies were harvested and stained with lacto phenol in cotton blue and viewed microscopically. The macro- and microscopic identifications of the species (Fig. 1) isolated from the compound feed study samples were done following the identification keys of Klich and Pitt (1988) and Klich (2002).

2.3 Molecular analysis

2.3.1 Fungal DNA Extraction

Isolates of pure fungal strains for DNA extraction were sub-cultured on yeast extract sucrose (YES) broth medium and incubated for 7 days at 25°C according to modified method of Fredlund *et al*. (2008). The extraction of DNA was performed using a DNA extraction Mini kit according to the manufacturer's (Qiagen White Scientific) modified protocol. The purified DNA was stored at -20°C until further analysis.

2.3.2 PCR reaction to amplify the aflD gene of aflatoxin-producing moulds

A 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 had 2.5 µl of DNA sample solution hich was mixed with 5 µl master mix (Taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplication 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

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eppendorf tubes placed in 36-well rack of the GeneAmp 5700R Sequence Detection RT-PCR System. Incubation proceeded for 2 mins at 50 ^{0}C to allow for cleavage of uracil-Nglycosylase. AmpliTaq Gold activation was done by incubating for 10 mins at 95 ^{0}C. The following temperature range of 95 ^{0}C for 20 s, 55 ^{0}C for 20 s and 72 ^{0}C for 30 s were used for the 35 PCR cycles.

2.3.2 Gel electrophoresis

Agarose gel DNA electrophoresis was performed according to the modified method of Saghai-Maroof *et al.* (1984). Two grams of agarose (BioRad agarose, Qiagen) was prepared in 98 ml 1x TAE (Tris/Acetate/EDTA) buffer to give a 2% solution and heated to boiling point in a water bath. The solution was allowed to cool to 60°C prior to the addition of 3 µl ethidium bromide (Et Br) (10 mg/l in water to a final concentration of 0.5 um/ml) and thoroughly mixed. The gel was poured in glass plates. Polymerase Chain Reaction products (as published in Iheanacho H.E. *et al*., (2014) were slowly loaded (2 µl) into the wells. A voltage of 5 V/cm was applied to the gel for the electrophoresis run and PCR product (Figure 2) was viewed using the Vacutec Gel documentation system and product size confirmed by comparison to the Middle Range Fast Ruler. The molecular size of DNA obtained after extraction was determined by gel electrophoresis. The gel electrophoresis allowed for the separation and visualization of DNA fragments from *A. parasiticus* and *A. flavus*.

3. RESULTS AND DISCUSSION

The occurrence and contamination levels of *A. flavus* and *A. parasiticus* in the various selected compound feeds were assessed and data summarised in Table 1. 1 A and B in Figure 1 show typical colonies of these species of fungi isolated from feed samples, while 1 C and D (Figure 1) show the morphological microscopic identification of each fungal species. Overall data indicated that 67.5 and 51.1% of feed samples were found to be contaminated with *A. flavus* and *A. parasiticus*, respectively. Accordingly, poultry feed had the highest contamination mean level when compared to cattle, and pig, while the lowest contamination was observed in horse feed.

A **B**

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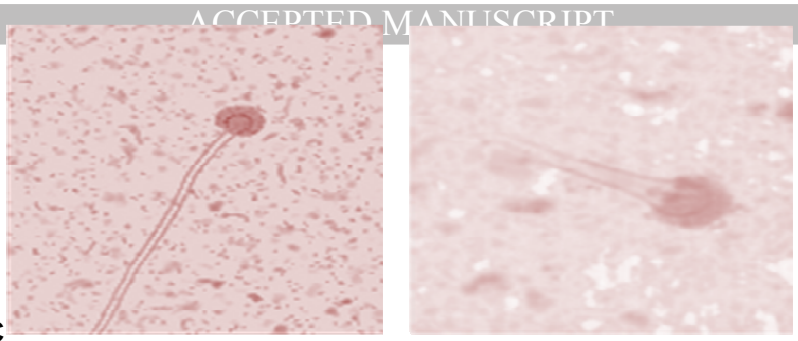

C D

Figure 1. Macroscopic (A & B) and microscopic (C & D) views of 6 day-old isolates of *Aspergillus flavus (A & C)* and *Aspergillus parasiticus (B & D) grown on* PDA

Table 1: Population of Aspergillus spp. (CFU/g) of compound feeds from South Africa

Feed types	N[a]	N[b]	Contamination level (CFU/g)
Poultry	62		
Layer	20	16 (80%)	4.0×10^{6}
Broiler	28	21 (75%)	5.7×10^{5}
Breeder	14	9 (64%)	3.4×10^{5}
Cattle	25		
Dairy	11	7(63.6%)	4.0×10^{4}
Dairy	11	7 (63.6%)	4.0×10^{4}
Calf	8	4 (50%)	3.8×10^{5}
Finisher	6	4 (66.6%)	3.5×10^{5}
Others			
Horse	3	1 (33.5%)	2.0×10^{3}
Pig	2	1 (50%)	2.7×10^{4}

a. Dilution ranged from 10-1 - 10-6

b. Na: Total number of feed type analysed

c. Nb: Positive/percentage contaminations

d. CFU/g: Colony forming unit per gram of sample

Reports from South Africa (Passone *et al.*, 2012, Somai and Belewa, 2011) and other regions of the world (Ige *et al.*, 2012, Pitt and Hocking, 2006, Razzaghi-Abyaneh *et al.*, 2006), show that the two species of *Aspergillus* are most widely studied. This maybe as a result of their occurrence frequencies, especially *A. flavus* as compared to *A. parasiticus* in feeds, and their individual abilities to produce aflatoxin. However, their identifications based on synoptic keys were compared to that obtained in this study.

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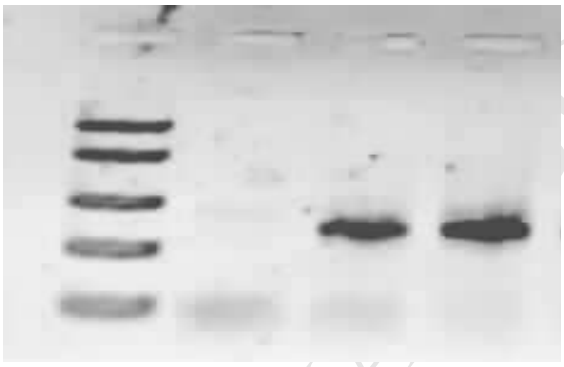
3.1 Molecular identification of *Aspergillus flavus* and *A. parasiticus*

Molecular sizes of the DNA of fungal species were estimated by the fluorescence intensity and comparison of the distance travelled with that of the molecular weight of marker standard as measured using gel electrophoresis and shown in Figure 2. However, the data indicated that the DNA fragment in lane IV compared to lane III has relatively distinct molecular sizes of 742 bp and 737 bp. This distinct size difference, in relation to their Restriction Fragment Length Polymorphism (RFLP) according to Somashekar *et al.*, 2004 studies, may be suggested to give reasons for the AF production capacity of *A. parasiticus* against that of *A. flavus* (Iheanacho, 2012, Iheanacho *ea tal*., 2014), with respect to the AF types they both produce.



i ii iii iv

Figure 2. Gel electrophoresis photo showing DNA portions of *Aspergillus* isolates. [i]: 5 bands molecular weight marker with different melting points, [ii]: Negative control (water), [iii]: *Aspergillus flavus,* [iv]: *Aspergillus parasiticus*[1]

Identification of fungi by molecular means is considered the most reliable over conventional method. Though it is expensive, labour and time intensive, it has become the most common tool for rapid identification *Aspergillus flavus*, *Aspergillus parasiticus* and other types of environmental fungi. The species of fungi identified are not morphologically and molecularly similar; however, they can be identified further as a variety of closely related species. Suggestions made by Martinez-Culebras and Ramon (2007), Varga *et al.* (2011) and El Khoury *et al.* (2011) on phylogenetic analysis using ITS and by Msiska (2008) and using <beta>-tubulin genes can be adopted in developing a differential relationship between closely related species of fungi like these. They two fungi initially identified morphologically appeared almost but not same species by molecular size. This suggests precise identification, looking at the presence of the nor-1 gene in these strains as closely related species in terms of DNA molecular size (600 kpb) which agrees with other reports (Mohankumar *et al.* (2010), Godet and Munaut (2010). However, molecular differentiation of A. flavus and A. parasiticus can be achieved by a detailed comparison of the restriction maps of PCR product of aflR-aflJ intergenic region fragment which allows identification of a restriction endonuclease, BglII (Feinberg and Vo-gelstein, 1984). Molecular methods have

[1] *DNA molecular weight marker Band sizes: 500 bp, 750 bp, 1000 bp, 1250 bp*

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been extensively useful in the identification of these *Aspergillus* species and several techniques such as random amplified polymorphic DNA analysis, DNA sequencing (Paterson 2006) and specific diagnostic PCR primers (Nicholson *et al.,* 1998) have been established for their systematic studies. However, by this study, *Aspergillus flavus* and *A. parasiticus* have shown to possess similar high degrees of DNA relatedness and genome size. They have virtually identical aflR gene (Chang *et al*., 1995) but based on their Restriction Fragment Length Polymorphism (RFLP), differentiation can be obtained (Somashekar *et al.*, 2004).

In dealing with animal feeds, correct as well as rapid identification of contaminating fungal species are important. It is also important to know if toxigenic fungi are present during pre- and post-production of feeds in order to rapidly employ the correct spraying administration. In the case of the fungal species under study, identification and differentiation is important because of the difference in their metabolite profiling, i.e. “B” and “G” type aflatoxins for *Aspergillus parasiticus* and “B” type only, for *A. flavus*. It is important to make mention herein that a number of other fungi belonging to the *Aspergillus, Penicillium*, and *Fusarium* species were also recovered as contaminants of feeds in this study as they have previously been isolated from livestock feeds (Logrieco *et al.,* 2003). *Aspergillus* species have most frequently been isolated from feed commodities kept under poor storage conditions (Pitt and Ailsa, 2009), i.e. aw of between 0.8 to 0.9 (Flannigan and Miller, 2001) with a wide temperatures range of 19-35 ℃ (Parra and Magan, 2004). These conditions favour growth of *Aspergillus flavus* and *A. parasiticus* to out-compete other fungi in stored products particularly in humid and hot climate regions like South Africa. The presence of *A. flavus* and *A. parasiticus* as observed in this study is in concordance with data reported by Mngadi *et al.,* (2008) for another set of feed in South Africa. However, in their study (Mngadi *et al.,* 2008), *A. parasiticus* was isolated from only two samples because none of the feeds contained peanuts which is a very suitable substrate for the growth of this fungus. In our study, the feed samples contained peanuts which gave high profile indices for the two fungal growths. These were also reported in similar works of Banu and Muthumary (2010) and Ouattara–Sourabie *et al.,* (2012) in some other countries. The two species of *Aspergillus* isolated in the present study ranked according to their isolation frequency. *Aspergillus flavus* was in abundance and more commonly recovered from samples than *A. parasiticus*. *A. flavus* is reported to be more widely distributed and have a higher occurrence frequency in agricultural commodities when compared to *A. parasiticus*, although they both occur more frequently in foods than some other fungal species. In European countries like Brazil Simas *et al.* (2007), Argentina Dalcero *et al.,* (1998) and Spain (Bragulat *et al.,* 1995, Abarca *et al.,* 1994) and Accensi *et al.* (2004), high frequency of occurrences in livestock feeds have been recorded for these *Aspergillus* species. Also, African countries like Ghana Kpodo *et al.,* (2000), Italy Giorni *et al.,* (2007), Pakistan Shah et al. (2008) and Algeria Riba *et al.,* (2008), high frequency was recorded, especially in livestock feeds formulated from cereals. This may be due to the fact that different cereals make up a major ingredient of livestock feeds, since the mycoflora of cereals is a reflection of *Aspergillus* fungal

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contamination in livestock feeds. These contamination occurrences of *Aspergillus* species including that in this present study may be due to high temperature tolerance and high humidity in their morphological and biochemical growths in these regions.

4. CONCLUSION

Aspergillus flavus and *A. parasiticus* are fungal species that are ubiquitous in nature. These two fungi in particular, have been isolated from a wide range of livestock feeds, especially those of cereal and nut origins (Saleemi *et al.,* 2010). They are considered amongst the most important pathogenic fungi that contaminant livestock feeds universally (Ghiasian and Maghsood, 2011). There have been several reports (Owino *et al.,* 2008; Akande *et al.,* 2006; Bennett and Klich, 2003; Dutton and Kinsey, 1996; Dutton and Westlake, (1985) from South Africa on contamination of agricultural commodities by *Aspergillus flavus* and *A. parasiticus*. The DNA extraction protocol described in the study provides a tool for total DNA isolation from the species of fungi and can also be used for other morphological forms of filamentous fungi. The assay, developed in this study, is proposed as a rapid and, easy morphological and molecular differential method to identify *A. flavus* and *A. parasiticus* species isolated from compound feed in South Africa. It will help in understanding the distribution of *A. flavus* and *A. parasiticus* in these feed products where vast numbers of isolates can be screened in a short time. It will underscore the importance of molecular techniques for fungal identification and determine accurate toxicological risks because toxic profile of each species could be different.

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Highlights

- Identified and Isolated fungi species most susceptible to compound feed contaminations
- Developed a molecular method for fungi identification
- Process analysis for a rapid routine check of compound feeds in the lab against contamination
- Process check to avoid the proliferation of the fungi growth on feed and food products

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