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PHYLOGENETICS OF AFLATOXIGENIC MOULDS AND PREVALENCE OF AFLATOXIN FROM IN-PROCESS WHEAT AND FLOUR FROM SELECTED MAJOR STORES WITHIN NORTHERN NIGERIA

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ABSTRACT
Aflatoxigenic strains that produce aflatoxins may be similar morphologically but vary genetically. Sequenced *aflR-1* gene from this work was used to study relatedness of aflatoxigenic strains. Yeast extract sucrose agar (YESA) supplemented with 0.3% cyclodextrin and 0.6 % sodium Desoxycholate (YCSD) was used to characterize aflatoxigenic moulds. Total aflatoxin content of the samples was determined using Enzyme Link immunosorbent assay (ELISA). Multiplex PCR was carried out on aflatoxigenic and some non aflatoxigenic moulds using the genes; *aflR-1, omt-A, ver-1* and *nor-1*. The *aflR-1* PCR products were sequenced and used for Basic Alignment Search Tool (BLAST) and to generate dendogram. While the raw wheat samples presented highest total aflatoxin range 0.6 – 49 µg/kg, major stores presented values of 0.50 – 28 µg/kg, higher than that obtained in-process samples (0.70 – 26 µg/kg). However, there was no significant differences ($p \geq 0.05$) between the major store total aflatoxin levels and that of in-process samples. Thirty seven (37 %) and 25 % of the wheat flour samples from in-process and major stores respectively exceeded the 10 µg/kg National Agency for Food and Drug Administration and Control, Nigeria (NAFDAC) recommended limit for this product. Thirty six (36 %) and 21 % prevalence of wheat and wheat flour samples respectively analyzed from this work was contaminated with total aflatoxins. The multiplex PCR response from this work has demonstrated that there was consistency in the banding pattern of aflatoxigenic strains with respect to the major aflatoxin biosynthetic genes from this study. BLAST of the assembled *AFL2T* gene sequences was found to have compatibility with that of standard *aflR-1* of NCBI Gene Bank that also created way of identifying the isolates. The bootstrap similarity matrix differentiated the isolates into two major clusters; sub-group and others assembled together indicating closer relatedness or general similarity. Some of the isolates varied greatly with visible mixtures of aflatoxigenic and non aflatoxigenic moulds and also random distribution of in-process and store isolates at different leaf nodes within the major clusters. Awareness and surveillance of aflatoxin levels by processors and regulatory bodies at raw material intake is advocated.

Keywords: Moulds, Aflatoxin, Gene, Dendogram

INTRODUCTION

Phylogenetic trees are useful in the fields of bioinformatics and systematics for determining relatedness and to infer most recent common ancestor. The search for inferred most recent common ancestor of descendants, similarity and difference between different strains containing similar gene sequences such as *aflR-1* using cluster statistical analysis tool like unweighted pair group method with arithmetic mean (UPGMA) (Chang, 2003) and bootstrapping has been reported (Shapir*a et al.,* 1996*)*.
Although there are over 25 reported enzymes and two regulatory proteins involved in aflatoxin biosynthesis (Batnagar *et al*., 2006), novel molecular identification of aflatoxigenic moulds was reported through four key genes. The gene *aflR-1* regulates structural genes; *omt-A, ver-1* and *nor-1* positively at level of transcription during biosynthesis of aflatoxins (Woloshuk and Prieto, 1998; Chang *et al.,* 2002*)*. Farber *et al.* (1997) earlier reported that the *aflr* protein can bind the promoter region of aflatoxin synthesis gene and activate *aflR* gene expression. This is the switching on the regulatory section for expression of genes involved in polykitide pathway. Aflatoxigenic moulds are strains of moulds that produce aflatoxins, a mycotoxin that poses health threat to consumers through contaminated foods and feeds. Aflatoxins are carcinogenic and immunotoxic (Bagheri, 2012). The toxin is the third leading cause of cancer worldwide having aflatoxin B_1 as group one carcinogen (WHO 2008). Amplification of the four key genes that code for enzymes involved in aflatoxin biosynthesis from wheat flour to study the occurrence of aflatoxigenic strains and the relatedness of the isolates is crucial. For this reason, the aim of this research was to generate a dendogram using the sequenced *aflR-1* gene to determine relatedness and also to determine the prevalence of total aflatoxins from in-process and selected major stores from northern Nigeria.

MATERIAL AND METHODS

Collection of sample of wheat flour

Flour samples were obtained from four selected wheat flour processors that consented to this study and major wheat flour distribution stores within Northern Nigeria. The study was cross sectional study. Raw material (Wheat Grain) samples (50 x 200g) were withdrawn from each lorry load during discharge into the storage silos. The milling protocols of each of the four selected

wheat flour mills was followed individually and 50 samples x 100g of wheat flour was withdrawn aseptically from the finished product lines. Major store wheat flour samples (50 x 200 g) were also collected aseptically.

Polyphasic Characterization of Aflatoxigenic Moulds from In-process and Major Store Wheat and Flour

Aflatoxigenic moulds was cultured using specific detection media, Yeast extract sucrose agar (YESA) modified with 0.3% cyclodextrin and 0.6% sodium desoxycholate (YCSD) according to the method described by Ordaz *et al*. (2003).

Aflatoxin Producing Ability of Aflatoxigenic Moulds Isolates

Representative aflatoxigenic moulds that presented beige ring on the modified yeast extract sucrose agar was cut out from the agar and was extracted with 70% methanol according to the methods of Baranitharan *et al*. (2015). The aflatoxin contents was assayed using ELISA technique to detect and quantify the total aflatoxins produced by species after 14 days incubation on YES agar modified with 0.3% m-β-cyclodetrin and 0.6% sodium Desocholate according to the methods of Latha *et al*. (2008). ELISA is a solid phase direct competitive immunoassay. Where antibody coated micro titer wells cross react with the four aflatoxin subtypes. When excess reactants are washed and required volume of substrate reagent and stop solution are added. The optical density (OD) of each micro well was generated by the micro titer plate reader using a 450nm filter.

Extraction of DNA from Aflatoxigenic *Aspergillus* spp

Pure submerged cultures of isolates from the aflatoxigenic *Aspergillus* sp and non aflatoxigenic isolates were lysed using lyses buffer, and their genomic DNA extracted according to the methods adopted by Niessen, (2007) and used as template for multiplex PCR.

PCR

Primers

A synthesized primer was obtained from Biolab. The primer *aflR-1* is a sequence of regulatory protein that amplifies the regulatory gene *aflR-1* which regulates structural genes at transcription level. The structural gene *omt-A* encodes the enzyme O-methyltransferase A where it steps in aflatoxin biosynthetic pathway through conversion of Sterigmatocystin to O-methylsterigmatocystin. The primer *ver-1* is a sequence of structural gene (versicolorin A) which encodes an enzyme that is directly involved in the conversion of versicolorin A to demethylsterigmatocystin. The primer *nor-1* amplifies norsolorinic acid gene that encode an enzyme norsolorinic acid ketoreductase along the polykitide pathway.

Table 1: Published primer nucleotide sequences for key aflatoxigenic genes

Gene	Nucleotide sequence	Amplification Target	Source
aflR-	F 5`-TAT CTC CCC CCG GGC ATC TCC CGG-3`		
	R 5`-CCG TCA GAC AGC CAC TGG ACA CGG-3`	1032bp	(Scherm *et al*., (2005)
omt-A-	F 5`GGCCCGGTTCCTTGGCTCCTAAGC-3`		
	R 5`CGCCCCAGTGAGACCCTTCCTCG-3`	797bp	(Scherm *et al*., (2005)
ver-1	F 5` ATGTCGGATAATCACCGTTTAGATGGC-3`		
	R 5`CGAAAAGCGCCACCATCCACCCCAATG-3`	537bp	(Scherm *et al*., (2005)
nor-	F 5`ACC GCT ACG CCG GCA CTC TCG GCAC-3`		
	R 5`GTT GGC CGC CAG CTT CGA CAC TCC G-3`	400-bp	(Scherm *et al*., (2005)

PCR Conditions

PCR was carried out in a 50 µl reaction mixture plus 100 ng of genomic DNA, with deoxynucleoside triphosphates at 200 µM. Primers was used at 1µM each, and reaction buffer (10 mM Tris-HCl [pH 9.0], 50mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, and 0.2 mg of gelatin per ml). Each reaction mixture was heated at 95°C/10 min before 2.5 µl of *Taq* DNA polymerase added. A total of 30 PCR cycles was run (one cycle being 1 min at 94°C for denaturation, 2 min at 65°C for primer annealing, and 2 min at 72°C for extension) and a 5-min final extension at 72°C was run on a programmable DNA thermal cycler

Gel Analysis of DNA

The PCR products was analyzed by gel electrophoretic documentation system (AAB Advanced American Biotechnology, Fullerton, CA, USA) according to the methods of Gashgari *et al*., (2010) on a 1% agarose gel stained with ethidium bromide. The different molecular weights of the bands were determined against a DNA standard (100bp DNA molecular weights ladder, Biolab).

DNA Sequencing and Homology Search

PCR amplification product of *aflR*-1 were cut out of the electrophoretic gel and purified with a High Pure PCR UltraClean PCR Clean up kit (MO-BIO, Carlsbad, USA) and sequenced using AMBI PRISM 3730 DNA sequencer (APLIED Biosystems, Foster City, USA). All the amplification products were sequenced in both directions. Sequences of the fragment *aflR-1* were assembled to yield the entire *AFL2T* gene sequence. The *AFL2T* sequences of each strain were aligned for homology searches with National Center Biotechnology Information (NCBI) standard gene BLAST program AspGD MultiGenome NCBI Search on the website http://www.aspergillusgenome.org/cgi-bin/compute/blast_clade.pl

Phylogenetic Determination of Relatedness of Isolates Based on *aflR-1* nucleotide Sequences Using Neighbour-joining Dendogram

The similarity level was evaluated using bootstrap analysis on the basis of Maximum Likelihood method following Tamura-Nei model (Tamura and Nei, 1993). The Phylogenetic evaluation was conducted by applying neighbour-joining program from the Molecular Evolutionary Genetics Analysis version 7.0 (MEGA7) package. Phylogenetic tree was generated by joining one leave node to the other. The percentage of trees in which the associated taxa clustered together is shown next to the branches.

RESULTS AND DISCUSSION

Range distribution of total aflatoxin level (µg/kg in wheat and wheat flour from in-process and major stores is represented in Table 1. Aflatoxin levels obtained from this research showed a peculiar trend; the high levels of total aflatoxin in the raw material which dropped during the in-process was probably due to dilution of the toxins into wheat flour matrix. The authors Oh-Kyung Kwon *et al*. (2004) have reported that there is decrease in the content of mycotoxins in flour when compared with the bran. The higher level of total aflatoxin levels in stored wheat flour than the in-process sample may have been due to the contamination of the process lines with aflatoxigenic strains coupled with the high moisture level of the finished product when leaving the factory. This favours the proliferation of aflatoxigenic strains when present and hence the higher toxin levels at major stores. In-process samples presented a minimum total aflatoxin range values of 0.5 – 33 and 0.50 - 10 µg/kg and maximum range values of 0.6 – 49

and 0.70 – 26 µg/kg for wheat and wheat flour respectively. Samples from major stores presented minimum 0.50 – 20 µg/kg and maximum aflatoxin range of 0.50 – 28 µg/kg in wheat flour. Major store samples presented higher aflatoxigenic moulds counts than in-process samples (table not shown). However, there was no significant differences ($p \geq 0.05$) between the total aflatoxin values in major store and in-process samples. Thirty seven (37 %) and 25 % of the wheat flour samples from in-process and major stores respectively exceeded the 10 µg/kg total aflatoxin levels recommended limit by National Agency for Food and Drug Administration and Control, Nigeria for this product. Generally, bran significantly ($p \leq 0.05$) contained higher aflatoxin ranges than wheat flour (table not shown). Thirty six 36 % and 21% prevalence of the wheat and wheat flour samples respectively analyzed from this work was contaminated with aflatoxins. The high prevalence of 21 % of aflatoxins in wheat flour was also attributed to the use of contaminated raw material and poor manufacturing practices such as flour spillages which were seen being heaped up, later sacked and kept aside for days before being recycled into the milling stream. This tends to introduce inestimable cultured aflatoxigenic strain and hence the total aflatoxin level in the finished products.

The result from this work has shown that there was consistency in the banding pattern of aflatoxigenic moulds (isolate 2,4,5,6,7,8,9,10,11 and 12) unlike non aflatoxigenic counterpart (isolates 1 and 3) with high level of aflatoxin biosynthetic genes variability (Plate 1). Aflatoxigenic *Aspergillus flavus and Aspergillus parasiticus* presented four PCR products with the genes; *aflR-1, omt -A, ver-1 and nor- 1* from this study. The complete four gene band patterns obtained in this study (Plate 1) confirms the positive response for aflatoxin production by the isolates tested using polymorphic beige ring. Other workers reported that genes involved in the aflatoxin biosynthetic pathway may form the basis for an accurate, sensitive and specific detection system using multiplex PCR for aflatoxigenic strains in grains and products (Thompson *et al*., 1997; Somashekar *et al*., 2004; Gashgari *et al*., 2010). The four gene patterns for aflatoxin-producing strains obtained in this study are also in line with earlier reports of Criseo *et al*. (2001). Latha *et al*. (2008) reported the absence of only a*flR-1* and *omt-1* genes confirms non aflatoxigenic strains. Lee *et al*. (2006) suggested for safety reasons that *aflR-1* gene presence could be used to detect the aflatoxin production by aflatoxigenic strains because of its immense regulatory actions before transcription of the structural genes. All the other three genes (*nor-1*, *ver-1*, *omt-1*) used in this study were structural genes in cluster in aflatoxin biosynthetic pathway that code for key enzymes in production of aflatoxins (Yu *et al.,* 2004).

Sequencing of the major aflatoxin biosynthetic genes crated an excellent way of identification and differentiating closely related microorganisms belonging to the same species and those that produces the same secondary metabolite such as aflatoxins. Sequences obtained from this research were assembled to yield the entire *AFL2T* gene sequences. Compatibility of the *AFL2T* gene (flank DNA sense and antisense of the gene) was found to march entirely with standard *aflR*-1 of NCBI Gene Bank.

Phenetic analysis of the twelve aflatoxigenic isolates using the bootstrap similarity matrix from the sequences of *AFL2T* gene bands generated two main clusters in an attempt to resolve variability among the strains (Fig. 1). Based on the model on which the dendogramme was generated, isolates that cluster together show closer relatedness while those that assemble on the same leave node indicated greater genetic relatedness. Though the isolates were similar morphologically, they all vary genetically along their clade positions. Assuming the dendogramme was rooted, one would have say that aflatoxigenic and non aflatoxigenic isolates that cluster together could have arisen from the same ancestral origin. None the less, at this point, one is attempted to believe that the non aflatoxigenic strains clustering with aflatoxigenic strains may have been aflatoxigenic but might have lost one or more key genes due to mutation as explained by other workers (Geisen, 1996; Cary *et al*., 2000; Venden *et al*., 2001; Tominaga *et al*., 2006). Some of the isolates varied greatly with visible mixtures of aflatoxigenic and non aflatoxigenic moulds at different leaf nodes within the major clusters. Similarly, there was also random mixture of in-process and store isolates with different genetic diversities at different leaf node clade positions.

Conclusion

This work has shown consistent quadruplex banding pattern with the aflatoxigenic mould key genes (*aflR-1, omt -A, ver-1* and *nor-1)* in aflatoxin biosynthesis. Some in-process and major store aflatoxigenic strains assembled on the same leaf node indicating greater genetic relatedness. Others differentiated into subgroups on neighbouring leaf node clade position and largely into two clusters indicating different genetic diversities.

Thirty six 36 % and 21% prevalence of the wheat and wheat flour samples respectively analyzed from this work was contaminated with aflatoxins. There is need for surveillance and control of aflatoxigenic moulds and aflatoxins before and during processing wheat into flour.

Table 1. Range distribution of total aflatoxin levels in wheat and wheat flour

In-process Samples		Major Stores Samples
Wheat (µg/kg)	wheat flour (µg/kg)	wheat flour (µg/kg)
M_A 0.60 - 49	0.50 - 23	S_A 0.50 - 26
M_B 0.50 - 45	0.70 - 26	S_B 0.50 - 20
M_C 0.50 - 33	0.60 - 15	S_C 0.60 - 25
M_D 0.50 - 41	0.50 - 10	S_D 0.50 - 28
M_E 0.55 – 48	0.47 - 18	S_E 0.50 – 24

Key; M_A - M_E, are mill in-process samples.
S_A - S_E, are major store samples ___ ____

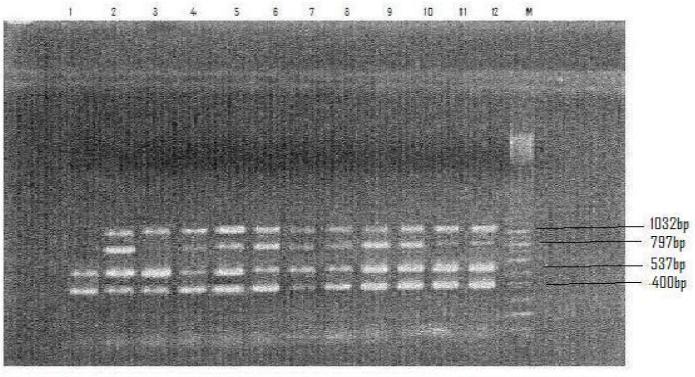

Plate 1: Electrophoregram of Multiplex PCR Products Obtained Using aflR-1,omt-A, ver-1 and nor-1 Genes from Aflatoxigenic Moulds Isolated from In-process and Major Stores.

Key: Lane 2,4-12 are Aflatoxigenic strains, Lane 1 and 3 are Non aflatoxigenic moulds, Lane M,Molecular size ladder (Biolab 100bp marker)

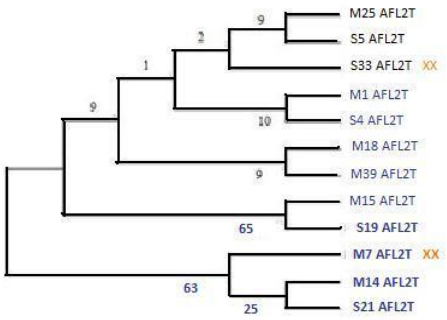

Fig. 1: Neighbour-joining Dendogram Using AFL2T Sequence Obtained from A.flavus and A.parasiticus
Key: M, mill isolate.,S, major store isolate., xx, non aflatoxigenic strains.,AFL2T, code for sequenced aflR-1

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