Profiles of aflatoxin biosynthetic key genes in non aflatoxigenic *Aspergillus flavus* isolated from in-process wheat flour from parts of Northern Nigeria

Wartu, J.R.'*., Whong, C.M.Z[2].., Abdullahi, I.O[3]. and Ameh, J.B[4].

[1234]*Department of Microbiology, Faculty of Life Sciences, Ahmadu Bello University, Zaria, West Africa Nigeria*

+2348037348233

Abstract: Aflatoxigenic moulds are strains that produce aflatoxins. Yeast extract sucrose agar supplemented with 0.3 and 0.6% beta methyl cyclodextrin and sodium desocholate respectively was used to discriminate between aflatoxigenic and atoxigenic strains. ELISA technique and multiplex PCR techniques was also used to further study aflatoxin producing ability of the strains and to detect the presence of aflatoxin key genes (*aflR-1, omt-A, ver-1* and *nor-1*) respectively. Isolates 1,2,3,4,5,6,7,8,11 and 12 were all atoxigenic strains. They all presented negative responds for polyphasic beige ring assay (cultural method) and also ELISA. On the other hand same isolates presented expected DNA fragments with varied banding patterns from 1, 2, and 3 each with different profiles of the key genes. None of the member isolates showed four band patterns with the aflatoxin biosynthesis key genes. While isolate 1, 2, 3, 8, 11 and 12 presented two band patterns each, isolate 4, 5 and 7 presented one band each. Isolate 6 three bands. Both the banding patterns had different profiles. Isolates 9 and 10 were aflatoxigenic strains and both isolates presented consistent four amplified banding patterns each with the aflatoxin biosynthesis key genes. Fifty (50.0%) of all the non-aflatoxigenic strain genes amplified in this study showed one profile, two DNA banding patterns. On the same hand, 66.7% of the two DNA amplification banding patterns showed varied profiles. Twenty (20.0%) of the non-aflatoxigenic strains showed one variable profile, one DNA amplification banding pattern. The aflatoxin key gene variable profiles was common to only non aflatoxigenic strains. Based on the key gene profiles, the gene *nor-1* had the highest frequency of occurrence (73.3%) followed by *ver-1* (53.3%), *omt-A* (40.0%) and least by *aflR-1* (13.3%).The variable PCR amplification of the key genes in non aflatoxigenic strain could be useful in ascertain the toxicological status of food products.

Key words: Gene, PCR, Aflatoxin

Introduction

Aflatoxin biosynthetic key genes are unit of DNA in aflatoxigenic moulds. The genes code for enzymes that are useful in the conversion of one substrate to the other along the aflatoxin biosynthetic pathway referred to as polykitide. There are over 25genes clustered within 70-kb DNA region involved in the polykitide pathway (Townsend, 1997). The polyketide-derived aflatoxins are produced through the conversion of acetate → polyketide → anthraquinones → xanthones → aflatoxins (Yu *et al*., 2002). However, four genes (*aflR-1, omt-1, ver-1* and *nor-1*) have been reported as the key aflatoxin biosynthetic genes. The detection of these key genes connotes that the strains could produce aflatoxins when appropriate conditions are met (Milani, 2013; Asurmendi *et al*., 2015; Amani *et al*., 2016). Aflatoxins are secondary metabolite of moulds strains; *Aspergillus flavus* and *Aspergillus parasiticus* (Yabe and Nakalima, 2004) that posed health hazards to both plants and animals. A wheat product such as wheat flour has particle size of 90 - 200µm which is a raw material for production of leavened bread, cakes, doughnuts etc. Semolina is also a wheat product with particles size 350-850 µm though the name varies from one processor to other. Brown flour contains a higher ratio of wheat flour than dusty wheat bran. Bran is actually the back covering of wheat grain mainly used for animal feed. The contamination of this products with aflatoxigenic strains and aflatoxins has been reported in wheat (Toteja *et al*., 2006; Taheri *et al*., 2012, Felaga *et al*., 2016), wheat flour (Fatemeh *et al*.,2013). Earlier Radiana *et al.* (2009) reported the concentration of aflatoxigenic moulds and aflatoxins on the bran and their introduction into wheat products is mostly through extraction (Alldrick, 1996; Schollen berger *et al*., 2002). Different molecular methods such as multiplex polymerase chain reaction (PCR) of the four key genes and Real-Time PCR have been used for the detection of aflatoxigenic strains. However, this study was aimed at detecting profiles of aflatoxin biosynthetic key genes in non aflatoxigenic strains of *A. flavus* isolated from wheat flour processed in parts of Northern Nigeria

Sample Collection and Preparation

Two hundred (200g) each wheat and wheat flour were collected from flour mill that operate within northern Nigeria once every day for two months. The samples were collected aseptically using sterile samplers and placed in sterile low density cellophane bags and were taken to the laboratory for analysis.

*Corresponding author:
reubjw@gmail.com* **Wartu, J.R.**[1]

Isolation and Polyphasic characterization of Aflatoxigenic *Aspergillus flavus*

Aflatoxigenic moulds was cultured using specific detection media Yeast extract sucrose agar (YESA) supplemented with 0.3% cyclodextrin and 0.6% sodium Desoxycholate (YCSD) according to the method of (C. laz *et al.*, 2003; Gashgari *et al.*, 2010; Alborch *et al.*, 2012). Sterile YCSD plates were prepared by adding 0.3% cyclodetrin and sterilized at 121^{0}C /15minutes. The modified medium was allowed to cooled to 45^{0}C before adding 0.6% sodium desocholate. The mixture were poured into sterile plates and allowed to gel. Serial dilution of each sample was made and aliquot measures of 0.1ml of the test samples were inoculated and spread using sterile bent glass rod on the sterile potato dextrose agar. The inoculated culture media plates were incubated at 27^{0}C for 7 days. All the *Aspergillus flavus* colonies were then inoculated into the modified YESA.

Aspergillus flavus that presented beige ring on the modified yeast extract sucrose agar was cut out from the agar and the aflatoxin extracted with 70% methanol according to the methods of Baranitharan *et al.* (2015). The aflatoxin contents was assayed using solid phase direct competitive ELISA technique to detect and quantify the total aflatoxins produced by species after 10 days of incubation on YES agar supplemented with 0.3% m-β-cyclodetrin and 0.6% sodium Desocholate according to the methods of Latha *et al.* (2008).

DNA extraction from *Aspergillus* spp

Pure submerged cultures of isolates from the aflatoxigenic *Aspergillus* sp and non aflatoxigenic isolates were lysed, and their genomic DNA extracted according to the methods adopted by El-Khoury *et al.* (2011) and used as template for multiplex PCR.

Determination of Aflatoxin Producing Ability of Isolates Using Elisa Technique

PCR

Primers

synthesized primer, produced using Prime 3` soft ware programme was obtained from Biolab.

Table 1: Published primer nucleotide sequences for key aflatoxigenic genes

Gene	Nucleotide sequence	Amplification Target	Source
*ver-1*F 5` ATGTCGGATAATCACCGTTTAGATGGC-3`			
R 5`CGAAAAGCGCCACCATCCACCCCAATG-3`	537bp	(Scherm *et al.*,(2005)	
omt-A-F 5`GGCCCGGTTCCTTGGCTCCTAAGC-3`			
R 5`CGCCCCAGTGAGACCCTTCCTCG-3`.	797bp	(Scherm *et al.*, (2005)	
*aflR-1*F 5`-TAT CTC CCC CCG GGC ATC TCC CGG-3`			
R 5`-CCG TCA GAC AGC CAC TGG ACA CGG-3`	1032 bp	(Scherm *et al.*, (2005)	
*nor-1*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 is at 95^{0}C/10 min before 2.5 µl of *Taq* DNA polymerase added. A total of 30 PCR cycles were run (one cycle being 1 min at 94^{0}C for denaturation, 2 min at 65^{0}C for primer annealing, and 2 min at 72^{0}C for extension) and a 5-min final extension at 72^{0}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 1 µg of ethidium bromide. The different molecular weights of the bands were determined against a DNA standard (100bp DNA molecular weights ladder, Biolab).

Results and Discussion

Cultural technique of moulds screening using modified YESA supplemented with β-methylcyclodextrin, PCR amplification response and aflatoxin detection using ELISA technique was carried

out on non aflatoxigenic strains js presented in (Table 1). Isolates 1,2,3,4,5,6,7,8,11 and 12 were all non aflatoxigenic. The isolates showed negative response for polyphasic beige ring assay and also ELISA. On the other hand same isolates presented expected DNA fragments with varied banding patterns 1, 2, and 3 each with different profiles (Plate 1). None of the isolates showed four band patterns with the key genes, but the deletion pattern of the key genes is common to non aflatoxigenic strains from this study. Results from Criseo *et al.*, (2001) indicated quadruple banding patterns in non aflatoxigenic strains besides the1,2 and 3 banding patterns from wheat isolates. This study didn't encounter isolates with quadruple pattern. The difference in the occurrence of quadruple pattern may be due to the different strains used. Also probably the quadruple atoxigenic strains could not produçe aflatoxins probably due to the key genes may have been defective and thus not expressed to produce aflatoxinsas explained by Criseo *et al.* (2008).This quadruple pattern in non aflatoxigenic strains has contradicted the variable key genes in non aflatoxigenic strains as the pointer to the toxicological status of food products. However, the use of transcript mRNA of the aflatoxin genes for identification of aflatoxigenic strains has also been suggested by other workers (Scherm *et al.*,2005).

Isolates amplification profile were as shown in plate 1; isolate 1(showed two bands amplification with *omt-A* and *nor-1*genes), isolate 2 (two bands amplification with omt-1 and ver-1genes), isolate 3 (two bands amplification with *omt-A* and *nor-1*genes), isolate 4 (one band amplification with *omt-A* gene),isolate 5 (one band amplification with bands amplification with omt-1 and nor-1genes ver-1gene), isolate 6 (three bands amplification with *aflR-1, omt-A* and *nor-1*genes), isolate 7 (one band amplification with nor-1gene), isolate 8 presented (two bands amplification with *omt-A* and *nor-1*genes), Isolate 9 and 10 (four banding patterns each with *aflR-1,omt-A,ver-1 and nor-1*), isolate 11 and 12 (two bands amplification with *ver-1* and *nor-1*genes). While isolate 1, 2, 3, 8, 11 and 12 presented two bands each, isolate 4,5 and 7 presented one band each. Both banding patterns had different profiles. Isolates 9 and 10 are aflatoxigenic strains used as internal control.

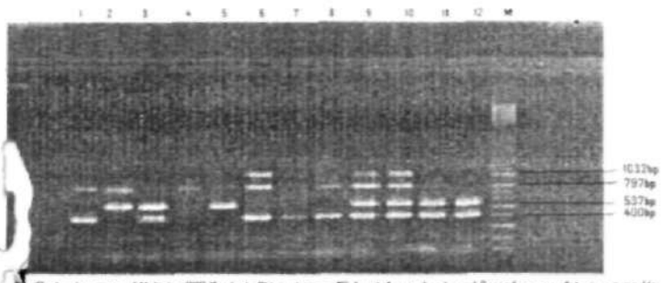
The DNA amplifications of isolates 9 and 10 (Plate 1) were that of beige ring aflatoxigenic strain presenting consistent four banding patterns each.

Fifty percent (50.0%) of all the non-aflatoxigenic strain genes amplified in this study showed one profile, two DNA banding patterns. On the same hand, 66.7% of the two DNA amplific banding patterns showed varied profiles. Tw percent (20.0%) of the non-aflatoxigenic strains sho one variable profile, one DNA amplification band pattern. The genetic variability was common to on non aflatoxigenic strains. The Frequency of occurren of key genes in aflatoxin biosynthesis indica variability among non aflatoxigenic strains is show figure 1. The genes from non aflatoxigenic stra showed that the *nor-1* gene had the highest frequency occurrence (73.3%) followed by *ver-1* (53.3%), *omt* (40.0%) and least by *aflR-1* (13.3%).The differ profiles of the key genes in this study showed hi aflatoxin biosynthetic genes [illegible] nonaflatoxigenic mould. Comparably the frequencies of occurrences obtained in this study were lower than those reported earlier by Criseo *et al.*, (2001) who reported *nor-1* (88%), *ver-1* (70%), *omt-A* (70%), and *aflR-1* (61.9%) from non-aflatoxigenic strains. This variability was earlier explained by other workers among who are (Geisen, 1996; Cary *et al.*, 2000; Tominaga *et al.*,2006; Maro *et al.*,2013 and Houshyarfard *et al.*, 2014) who suggested that different types of mutations could have been responsible for inactivating the aflatoxin biosynthetic pathway of the non aflatoxigenic moulds. On the same hand Criseo *et al.* (2008) attributed this variable profiles to defects on various molecular levels such as post transcriptional level or protein.

Table 1: Conventional and PCR Based Moulds Screening and Aflatoxin Detection Using ELISA Technique on non Aflatoxigenic Strains.

Strain code	Beige Ring on Modified YESA	Aflatoxin Response Using ELISA technique	PCR amplification response Using biosynthetic genes			
			aflR-1	*omt-1*	*ver-1*	*nor-1*
1	-	-	-	+	-	+
2	-	-	-	+	+	-
3	-	-	-	-	+	+
4	-	-	-	+	-	-
5	-	-	-	-	+	-
6	-	-	+	+	-	+
7	-	-	-	-	-	+
8	-	-	-	+	-	+
9	+	+	+	+	+	+
10	+	+	+	+	+	+
11	-	-	-	-	+	+
12	-	-	-	-	+	+

Key: +, Posive Response -, Negative Response



Electrophorogram of Multiplex PCR Products Obtained using aflR-1 and A. ver-1 and nor-1 Genes from non aflatoxigenic moulds (Lane 1 -12: Isolate codes (1-8, 11 and 12 are non aflatoxigenic Moulds while Lane 9 and 10 are aflatoxigenic strains) Lane M:Molecular size ladder (Bioneer 100bp Marker)

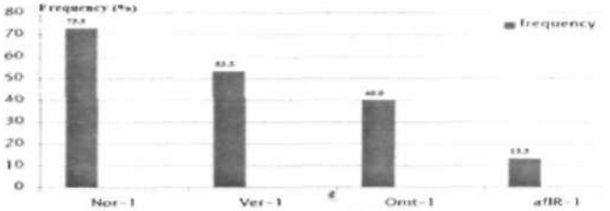


Fig. 1: Frequency of occurance of key genes in aflatoxin biosynthesis indicating variability among non Aflatoxigenic Moulds

Conclusion

To the extent of this study, aflatoxigenic key genes had been indexed from non aflatoxigenic isolates of *Aspergillus flavus* from wheat flour, processed in factories in northern Nigeria

The non aflatoxigenic *A. flavus* showed one, two and three PCR amplification banding patterns with different profiles. While fifty (50.0%) of all the *A. flavus* used in this study showed PCR amplification of one profile, two DNA banding patterns, 66.7% of the two DNA amplification banding patterns showed varied profiles. Only twenty (20.0%) of the non-aflatoxigenic strains showed one variable profile, one DNA amplification banding pattern.

The genetic variable profiles were common to only non aflatoxigenic strains. The Frequency of occurrence of key genes were in this order: *nor-1* (73.3%) >*ver-1* (53.3%) >*omt-A* (40.0%) and least by *aflR-1* (13.3%). The non aflatoxigenic *Aspergillus flavus* could be used to ascertain toxicological status of food products since the different key gene profiles generating variability is common to atoxigenic strains.

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