

Original Research Article

Antibiogram, Molecular Characterization and Detection of Aflatoxigenic Gene from some Species of *Aspergillus* Cereals

Abstract

Aflatoxins are potent mycotoxins produced by certain strains of *Aspergillus*, which pose significant threats to human and animal health due to their carcinogenic and mutagenic properties. The study aimed to determine the antibiogram, molecular characterization and detection of aflatoxigenic gene from some species of *Aspergillus* cereals. Seventy-two samples of maize, rice, wheat and millet were bought from different vendors in Mile 3, Mile 1 and RumuokoroMarket in Port Harcourt, Rivers State, Nigeria. The molecular characterization and detection of aflatoxin regulatory gene was achieved using a PCR based technique. ~~The isolates were screened for their susceptibility to antifungal agents using the disc diffusion method.~~ Data showed the isolates were *Aspergillus flavus* strain HBF576, *Aspergillus flavus* strain AS25, *Aspergillus niger* strain A40, *Aspergillus flavus* strain 64-A1, *Aspergillus flavus*, A1S6_8 and *Aspergillus flavus* strain AKF-10. More so, thirteen (86.7%) of the fungal isolates had the aflatoxin regulatory gene. All five isolates of *A. niger* had the aflatoxin regulatory gene while only eight of the *A. flavus* possessed the gene. The antibiogram showed that 100% of *A. flavus* and *A. niger* were completely susceptible to nystatin while 95.45% of *A. flavus* and 100% of *A. niger* were susceptible to itraconazole. Ketoconazole was the least potent antifungal agent. The presence of aflatoxin-producing *Aspergillus* bearing the aflatoxin regulatory gene could be a concern to the public especially with the documented effect of aflatoxin on human health. Furthermore, the nystatin which displayed high potency on the fungal isolates is recommended for use in the control of these isolates.

Keywords: aflatoxigenic gene, antibiogram, *Aspergillus*, cereals, molecular characterization,

1.0 INTRODUCTION

Cereals and cereal products constitute a large portion of food resources and are consumed by a large number of people worldwide [1]. The cereals are annual common grass members of the grass family such as wheat, rice, maize, sorghum, millet, barley and rye, whose starchy grains are used as food [2]. Cereals and derived products represent an important nutrient source for mankind worldwide. In addition, they are the most important dietary food for North African populations [3]. They are the major source of calories and proteins for the people of Nigeria and the major cereals cultivated in

Comment [TM1]: Data showed that they were identified as *A. flavus* and *A. niger*.

Comment [TM2]: In the PCR result of aflR, the isolate IHB which phylogenetically identified as *A. niger* (Figure 1) showed negative result. Please clarify.

Comment [TM3]: Please add the introduction on the "Antibiogram"

Nigeria are sorghum, millet, rice and maize. Cereal grains constitute an important group of substrates for fermented foods in tropical Africa [4]. The major cereal crops in Nigeria are rice, maize, sorghum, wheat, millet, sugar cane and fonio millet with rice ranking as the sixth major crop in terms of land area while sorghum accounts for 50% of the total cereal production and occupies about 45% of the total land area devoted to cereal production in Nigeria [5]. In West Africa, crop production is mostly concentrated in the drier parts of the region owing to its drought tolerance. It is mainly grown in the Northern areas of Nigeria and the Republic of Niger [6].

Unfortunately, cereals are naturally contaminated with fungi in the field, during drying, processing, transportation and subsequent storage and it may be difficult to completely prevent mycotoxin formation in contaminated commodities, particularly those that are produced in tropical and subtropical climates, in countries where high temperature and humidity promote the growth and proliferation of fungi [7]. Thus, they are often colonised by fungi, including species from the genus *Aspergillus*, *Penicillium* and *Fusarium*, which cause significant reductions in crop yield, quality and safety due to their ability to produce mycotoxins [4].

Mycotoxins are toxic secondary metabolites produced by filamentous fungi in a wide range of agricultural commodities worldwide, including cereals, nuts, legumes, spices, fruits, and their products [5]. The mycotoxins commonly occurring in cereals and cereal products include zearalenone, fumonisins, trichothecenes (as deoxynivalenol and T2-HT2), ochratoxin and aflatoxins [8]. Among various types of mycotoxins, aflatoxins (AFs) are highly toxic and are known to contaminate a wide variety of foods such as maize, groundnuts, dried fruits, meat and milk-based products [9]. Aflatoxins are naturally occurring mycotoxin that is largely produced by *Aspergillus flavus* and *Aspergillus parasiticus* species of fungi. This is a highly toxic secondary metabolite that contaminates several crops, causing a great economic loss [10]. It was reported that 25-50% of harvested world crops have been contaminated with mycotoxins and also about 50-80% damage to farmers' grain during the storage period or favourable conditions which causes significant loss both quantitatively and qualitatively is caused by fungi [4]. The storage fungi damage the grains in several ways: reducing the germinability,

producing undesirable odour and kernel discolouration, depletion in seed viability, hardness, colour, size and shape, grain weight and various biochemical parameters; protein, carbohydrate and vitamins decrease the food value and also produce toxins injurious to the health of consumers [11]. Many studies have used phenotypical methods in characterizing fungal isolates associated with cereals. This study however, evaluated the antibiogram and presence of aflatoxigenic gene markers of fungal isolates in cereals. The data obtained will be useful in deciphering the potential hazards associated with cereals in Port Harcourt, Rivers State, Nigeria.

2.0 Materials and Methods

2.1 Collection of Samples

The study was a cross-sectional study and adopted a systematic random sample approach of which each sample from different vendors had equal chance and numbers within the sample frame. The sampled locations used included three major Markets in Port Harcourt: Mile 1, Mile 3 and Rumuokoro markets. The choice of these markets was based on the fact that the grains were mostly sold there both in wholesale and retail quantities. Also, these markets are situated in different locations in Port Harcourt. The study was conducted from December 2022 to June 2023.

Seventy-two (72) samples comprising maize, rice, wheat and millet were bought randomly from two different vendors in each market, based on prevalence data by previous researchers [12].

2.2 Isolation of fungi

Please provide the method for isolation of fungi from cereal samples.

2.2 Molecular Identification of Fungal Isolates

Extraction of Fungal DNA

Extraction was done using a ZR fungal DNA prep extraction kit. A heavy growth of the spores of the investigated isolate was suspended in 200 μL of isotonic buffer into a ZR Bashing Bead Lysis tubes and 750 μL of lysis solution was added to the tube. The tubes were secured in a bead beater fitted with a 2ml tube holder assembly and processed at maximum speed for 5 minutes. The ZR bashing bead lysis tubes were centrifuged at 10,000 $\times g$ for 1 minute. Four hundred μL of supernatant was transferred to a Zymo-Spin IV Filter (orange top) in a collection tube and centrifuged at 700 $\times g$ for 1 minute. One thousand two hundred μL of fungal/ bacterial DNA binding buffer was added to the filtrate in the collection tubes bringing it to a final volume of 1600microlitre. Eight hundred μL was then transferred to a Zymo – spin IIC column in a collection tube and centrifuged at 10,000 $\times g$ for 1 minute and the flow through was discarded from the collection tube. The remaining volume was transferred to the same Zymo- spin and spun.

Two hundred μL of the DNA pre- washed buffer was added to the Zymo- spin IIC in a new collection tube and spun at 10,000 $\times g$ for 1 minute followed by the addition of 500 μL of fungal/ bacterial DNA wash buffer and centrifuged at 10,000 $\times g$ for 1 minute. The Zymo- spin IIC column was transferred to a clean 1.5 μL centrifuge tube. Onehundred μL of DNA elution buffer was added to the column matrix and centrifuged at 10,000 $\times g$ for 30 seconds to elute the DNA. The DNA concentration was quantified using the Nanodrop 1000 spectrophotometer. The ultra-pure DNA was then stored at -20°C for other downstream reactions [13].

Quantification of the Fungal DNA

~~The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was launched by double clicking on the Nanodrop icon. The equipment was initialized with 2 μl of sterile distilled water and blanked using normal saline. Two microlitre of the extracted DNA was loaded onto the lower pedestal and the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the “measure” button [13].~~

Amplification of 48-ITS Region

The ITS region of the rRNA genes of the isolates was amplified using the ITS4: 5'-TCCTCCGCTTATTGATATGS-3' and ITS5: 5'-GGAAGTAAAAGTCGTAACAAGG-3', primers on ABI 9700 Applied Bio systems thermal cycler at a final volume of 30 μ L for 35 cycles. The PCR mix included: X2 Dream taq Master mix (taq polymerase, dNTPs, MgCl), the primers at a concentration of 0.4 M and the extracted DNA as a template. The PCR conditions were as follows: Initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing at 53°C for 30 seconds, extension at 72°C for 30 seconds for 35 cycles and final extension at 72°C for 5 minutes. The product was resolved on 1% agarose gel at 120V for 15 minutes and visualized on a blue light transilluminator [13].

Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer. The sequencing was done at a final volume of 10 μ l, the components included 0.25ul BigDye terminator v1.1/3.1, 2.25 μ L of 5 x BigDye sequencing buffer, 10uM primer, PCR primer and 2-10ng PCR template per 100bp. The sequencing condition was as follows: 32 cycles of 96°C for 10seconds, 55°C for 5seconds and 60°C for 4 minutes [13]

Phylogenetic analysis

Obtained sequences were edited using the bioinformatics algorithm. Similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTIN. These sequences were aligned using Clustal X. the evolutionary history was inferred using the Neighbor- Joining method in MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes- Cantor [13]

Amplification of Aflatoxin Regulatory Gene

For the amplification of clustered pathway genes in aflatoxin biosynthesis, the PCR reaction was performed following the method described by Rahimi *et al.* (2016). The aflR – F 5'- AGAGCTACTGAACGTCCCAT-3' 1458 60 and aflR – R 5'- ATCAGGTTGCACGAACTGTCC-3' primer was used. The genes were amplified by

separate reactions following optimization. For this reason, the PCR mixture was prepared by adding 150 ng of DNA template, 1.5 mM MgCl₂, and 10X PCR buffer containing 50 mM KCl, 1 mM dNTP, 2.5 U of taq polymerase, and 0.3 pmol of each primer, and then reached to 50 µL with distilled water. The PCR products were electrophoresed on agarose gel (1%), stained with ethidium bromide, and visualized under ultraviolet light using a gel documentation system

Antifungal Susceptibility Testing

All *Aspergillus* species isolated from the samples were subjected to in-vitro antifungal susceptibility test by Kirby-Bauer disc diffusion method as recommended by CLSI [14].

Stock inocula were prepared by adding 24 hours old cultures to 5mL sterile normal saline. The culture was adjusted to a 0.5 McFarland's standard to obtain final inoculum volume of 2.0×10^5 CFU/mL. After which, sterile swabs were used to spread the test

isolates on the surface of well labelled freshly prepared dried SDA plates. Plates were allowed to dry before discs containing the antifungal agents were placed aseptically and incubated. After incubating the plates at 22-25°C for 24-48 hours, the plates were observed for zone of inhibition which was measured using a graduated rule. Isolates inhibition zones less than 8 mm were considered to be susceptible the antifungal agents.

Comment [TM4]: Please check this procedure, the stock inoculum was made from the spore suspension. The unit of inoculum concentration should be "spores/ml".

3.0 Results

Please inform that how many fungal isolates were recovered from cereal samples. Then, six isolates were subjects to molecular identification by ITS sequencing. How many isolates were further screened for the presence of the aflatoxin regulatory gene?

3.1 Molecular Characterization of the Isolates

Results of the agarose gel electrophoresis showing the amplification of the internal transcribed spacer (ITS) showed that all the six fungal isolates were within the 650bp for fungi identification (Plate 1)

The results of the phylogenetic tree of the fungal species showed that the representative fungal isolates had a high similarity index with those in the NCBI database and were identified as *Aspergillus flavus* strain HBF576, *Aspergillus flavus* strain AS25, *Aspergillus niger* strain A40, *Aspergillus flavus* strain 64-A1, *Aspergillus flavus* isolate A1S6_8 and *Aspergillus flavus* strain AKF-10 (Fig. 1).

Results of the accession number of the isolates and percentage similarity is presented in Table 1.

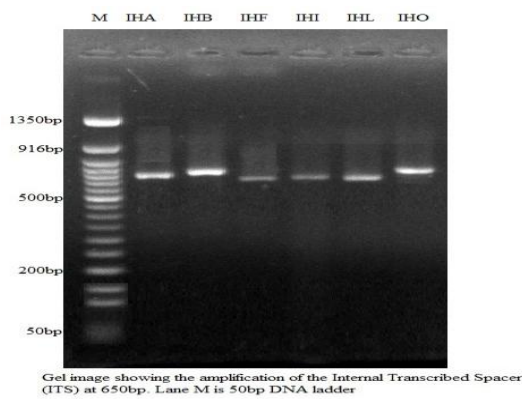


Plate.1: Agarose gel electrophoresis output of the amplicons

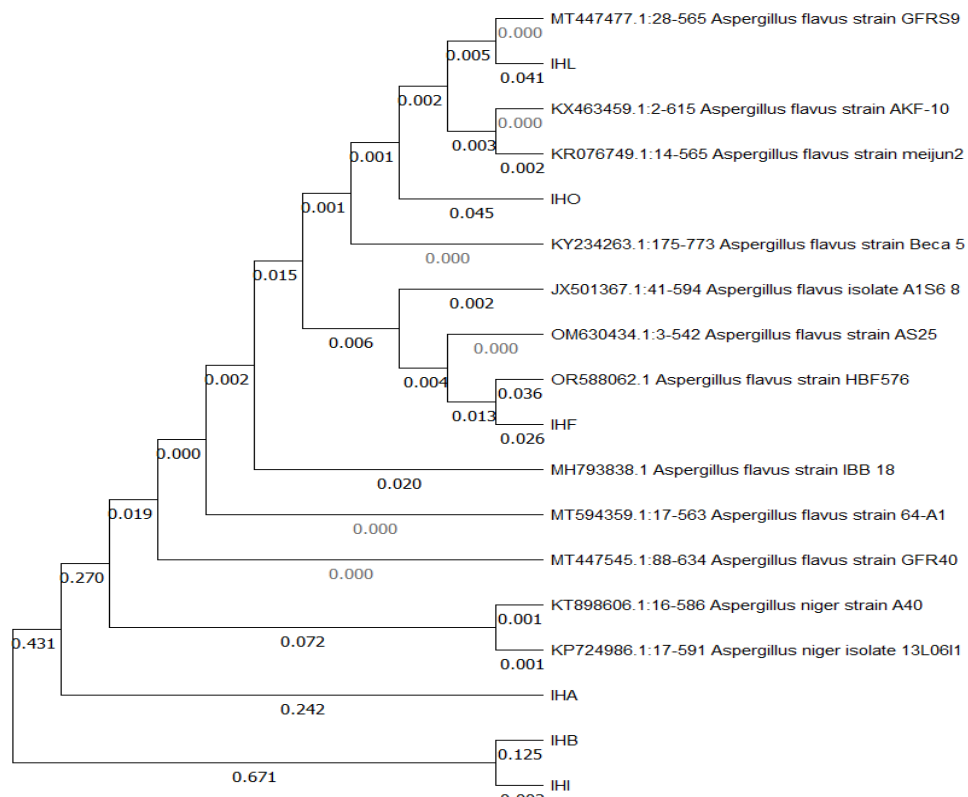


Fig. 1: Evolutionary Relatedness of the Fungal Isolates

Table 1 Accession number of the isolates

Phenotypic Identity	Molecular Identity	Accession Number	% Similarities
<i>A. flavus</i>	<i>Aspergillus flavus</i> strain HBF576	OR588062	93.45
<i>A. flavus</i>	<i>Aspergillus flavus</i> strain AS25	OM630434	93.50
<i>A. niger</i>	<i>Aspergillus niger</i> strain A40	KT898606	100
<i>A. flavus</i>	<i>Aspergillus flavus</i> strain 64-A1	MT594359	99.82
<i>A. flavus</i>	<i>Aspergillus flavus</i> isolate A1S6_8	JX501367	95.85

<i>A. flavus</i>	<i>Aspergillus flavus</i> strain AKF-10	KX463459	94.15
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Results of the agarose gel electrophoresis for aflatoxigenic gene (Aflatoxin regulatory gene) markers in Plate 2 showed that thirteen (86.7%) of the fungal isolates had the aflatoxin regulatory gene while two (13.3%) did not.

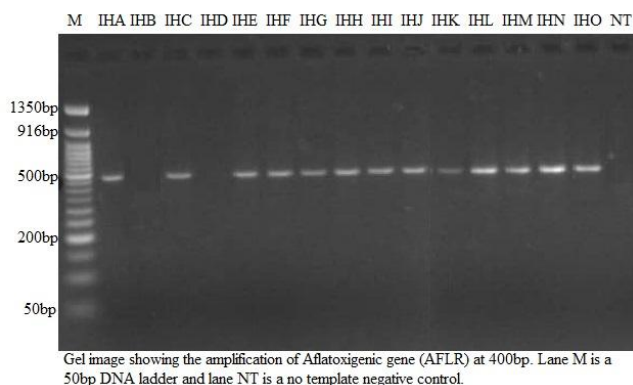


Plate 2: Agarose gel electrophoresis for aflatoxin gene markers

3.2 Antifungal Susceptibility of the Isolates

The results of the response of the isolates to the various antifungal drugs is presented in Table 2. Results showed that the isolates had varying responses to the antifungal agents. Results further showed that 88.57% of the fungal isolates were susceptible to ketoconazole, while 11.43% of the isolates were resistant (not inhibited). The Itraconazole antifungal agent showed better antifungal activity than ketoconazole with 97.14% of the isolates susceptible while 2.86% showed resistance. More so, the % susceptibility of the fungal isolates to nystatin was 100% while the % susceptibility to fluconazole was 2.86%.

Table 2: Response of the fungal isolates to the conventional antimycotic drugs used for *A. flavus* and *A. niger*

	<i>A. flavus</i> n =22		<i>A. niger</i> n =13	
Antifungal (5mg/mL)	Resistance n (%)	Susceptibility n (%)	Resistance n (%)	Susceptibility n (%)
Fluconazole	21 (95.45)	1 (4.55)	13 (100)	0 (0)
Itraconazole	1 (4.55)	21 (95.45)	0 (0)	100 (13)
Ketoconazole	4 (18.18)	18 (81.82)	0 (0)	100 (13)
Nystatin	0 (0)	22 (100)	0 (0)	100 (13)

Keys: n = number of isolates

4.0 Discussions

Genomic characterization showed the fungal isolates that frequently contaminated the grains vended markets sampled were *A. flavus*, followed by *A. niger*. ~~Aspergillus flavus strain HBF576, Aspergillus flavus strain AS25, Aspergillus niger strain A40, Aspergillus flavus strain 64-A1, Aspergillus flavus isolate A1S6_8 and Aspergillus flavus strain AKF-10.~~ The fungal isolates (*A. niger* and *A. flavus*) in the present study have been reported to be associated with grains. Krnjaja *et al.*, [15] reported the presence of *Fusarium*, *Aspergillus* and *Penicillium* sp in their study. The present study however, is in agreement with findings of Shamsuddeen *et al.*, 2017 [16] who also isolated *A. flavus* and *A. niger* amongst other fungal isolates in grains.

One of the most important mycotoxins that accompanies different types of diseases including cancers is aflatoxins [17]. In the present study, aflatoxin gene markers were detected in some of the isolates. Out of the fifteen isolates screened molecularly for the aflatoxin regulatory gene (aflR), thirteen (86.7) of the isolates were positive for the gene while two (13.3%) were negative and among these isolates, all the five *A. niger* and eight *A. flavus* isolates were positive for the gene while two of the *A. flavus* lacked the gene. The aflR gene regulates the activity of other structural genes in the aflatoxin biosynthesis pathway. On the other hand, aflD (Nor-1) gene plays an essential role in the early conversion of norsolorinic acid into averantin. The aflP is involved in the conversion of sterigmatocystin into aflatoxin in the late steps of the aflatoxin

Comment [TM5]: These strains are the results of BLAST analysis that showed similarity to your *A. flavus* and *A. niger* isolates.

Comment [TM6]: This sentence is quit long, please rephrase. In addition, *A. niger* isolate IHB showed a negative results for the PCR of aflR. Please clarify this results.

biosynthetic pathway [17]. The presence of aflR gene in these isolates could imply their ability to synthesize aflatoxins and the presence of aflatoxins in food could become harmful to consumers. Previous studies have reported that aflatoxins have teratogenic, mutagenic, and carcinogenic effects and their presence in human food chain may be a threat to the public health [18, 19]. Amaike and Keller [20] have reported that different species of *Aspergillus* such as *A. flavus*, *A. parasiticus* and *A. nomius* are aflatoxin producing fungi. This corroborates with the present study which showed that most of the *A. flavus* possessed the aflR. Khalid *et al.*, [21] reported 79% prevalence of aflR gene in 31 strains of *A. flavus* while in the present study, 86.7% from thirteen isolates was recorded. More so, the presence of the aflR gene in *A. niger* might not necessarily imply that they could all produce aflatoxins (Khalid *et al.*, 2018). Eramiet *al.*, 2007[22] and Rashid *et al.*, 2008[23] also signified the importance of three structural genes: nor-1, omt-1, and ver-1 and one regulatory gene, that is, afl-R in aflatoxin biosynthesis cluster and associate their presence with aflatoxin production. In line with previous studies, the absence of aflatoxin production in some aflatoxigenic species could be due to the deletion and simple mutation (replacement of some bases) of aflR gene or loss of other responsible genes in aflatoxin production pathway. However, some physiological conditions could also affect aflatoxin biosynthesis [24].

The findings of the antifungal activity showed that all the isolates of *Aspergillus* (*A. niger* and *A. flavus*) were all inhibited by nystatin while itraconazole was the second most effective antifungal agent inhibiting 97.14% of the *Aspergillus* isolates. The present study also showed that fluconazole was the least effective antifungal agent and was only able to inhibit the proliferation of 2.86% of the fungal isolates. More so, the present study showed that all the azole antifungal agents did not inhibit all the fungal isolates despite some (itraconazole and ketoconazole) showing some level of antifungal activity against a high proportion of the fungal isolates. Azole-resistant *Aspergillus* infections in many countries well documented. Previous study attributed fungal resistance to azoles as a single molecular resistance mechanism consisting of a 34-bp tandem repeat TR34

in the promotor region of the azole target CYP51A gene and a point mutation in the target gene itself leading to an L98H amino acid substitution (TR34/L98H) [25]. The broad ability of nystatin antifungal agent to completely inhibit the growth of all the *Aspergillus* species could be attributed to its broad spectrum of activity. Jalil *et al.*, 2020 [26] in their study reported that nystatin completely inhibited the growth of *A. niger*, *A. fumigatus*, and *Ochracioussp* due to its broad-spectrum activity both in-vitro and in-vivo. The mechanism of action of nystatin has been attributed to binding to ergosterol in fungal membranes resulting in an altered membrane permeability [27].

4.0 Conclusion

The species of fungi such as ~~*Aspergillus flavus* strain HBF576, *Aspergillus flavus* strain AS25, *Aspergillus niger* strain A40, *Aspergillus flavus* strain 64-A1, *Aspergillus flavus* isolate A1S6_8 and *Aspergillus flavus* strain AKF-10~~ identified in the present study could be a public health threat especially as some of the strains have been reported to be associated with diseases as well as production of mycotoxins (especially the isolates that possessed the aflR gene) that could be carcinogenic. Mores so, nystatin and itraconazole which were the most potent antifungal agents could be used in the control of infections resulting from *A. niger* and *A. flavus*.

Comment [TM7]: According to the molecular analysis, the isolates used in this study named IHL, IHO, IHF, IHF, IHB, and IHI. When the author mentioned on the isolates should be *A. flavus* strain IHL..., do not use the reference strains obtained from the BLAST analysis.

Comment [TM8]: Please specify the disease.

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