Original Research Article

Antibiogram andMolecular Detection of Aflatoxigenic Gene from some Species of *Aspergillus*in Cereal Grains

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 therefore targeted theantibiogram and molecular detection of aflatoxigenic gene from some species of Aspergillusin Cereal grains. Seventy-two samples of maize, rice, wheat and millet were bought from different vendors in Mile 3, Mile 1 and Rumuokoro Market in Port Harcourt, Rivers State, Nigeria. The molecular characterization and detectionof the aflatoxin regulatory gene was achieved using a PCR-based technique. The disc diffusion method was used in determining the antibiogram of the isolates. Data showed that the species were closely related to*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.Four 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 washowever, 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.The study indicated a high *in vitro* growth inhibition activity of nystatin, which could therefore serve as a potent antifungal agent in the control and management of infections associated with these fungal isolates in cereal grains.

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 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].

An antibiogram is a chart that displays the microorganisms' susceptibility to the tested antibiotics [12] or antimicrobial drugs.Due to the increasing rate of food borne infection associated with fungal contamination of grains, a continuous use of antifungal drugs is seemingly inevitable. This has however led to some form of drug resistance in fungal species.

Many studies have used phenotypicalmethods in characterizing fungal isolates associated with cereals. This study, however, evaluated theantibiogram and presence of aflatoxigenic gene markers of fungal isolates in cereal grains. 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].

2.1.1 Isolation of the fungal contaminants

The*Aspergillus species*were isolated from the cereal grains usinh the spread plated techniques, as described by Jallow *et al*., 2018[13]. In this method, an aliquot of 10^{-2} dilutions of each sample was inoculated into freshly prepared dried SDA plates supplemented with (200mg/l) Chloramphenicol in duplicates. Plates were labelled according to the samples, and aseptically spread using a sterile bent glass rod in the presence of a burner flame. The inoculated plates were incubated at 22-25°C for 3-7days. After incubation, the plates were observed for fungal growth. The spores of the fungal isolates were subcultured on newly prepared SDA plates and incubated at 25°C for 3 days. Microscopy and morphological appearance were adopted in fungal characterization before subjecting them to molecular methods.

2.2 Molecular Identification of Fungal Isolates

Extraction of Fungal DNA

Extraction was done using a ZR fungal DNA prep extraction kit. Heavy growth of the spores of the investigated isolate was suspended in 200 microlitres of isotonic buffer into a ZR Bashing Bead Lysis tube and 750 microlitres 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 xg for 1 minute. Four hundred (400) microlitres of supernatant were transferred to a Zymo- Spin IV Filter (orange top) in a collection tube and centrifuged at 700xg for 1 minute. One thousand two hundred (1200) microlitres of fungal/ bacterial DNA binding buffer was added to the filtrate in the collection tubes bringing it to a final volume of 1600 microlitres. Eight hundred (800) microlitres was then transferred to a Zymo – spin IIC column in a collection tube and centrifuged at 10,000xg 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 (200) microlitre of the DNA pre-washed buffer was added to the Zymo-spin IIC in a new collection tube and spun at 10,000xg for one minute followed by the addition of 500 microlitre of fungal/ bacterial DNA wash buffer and centrifuged at

10,000xg for 1 minute. The Zymo- spin IIC column was transferred to a clean 1.5 microlitre centrifuge tube. 100 microlitre of DNA elution buffer was added to the column matrix and centrifuged at 10,000 xg microlitre for 30 seconds to elute the DNA. The ultra-pure DNA was then stored at -20 degrees for other downstream reactions [14].

Amplification of 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 microlitres 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 95^0C for 5 minutes, denaturation at 95^0C for 30 seconds, annealing at 53^0C for 30 seconds, extension at 72^0C for 30 seconds for 35 cycles and final extension at 72^0C for 5 minutes. The product was resolved on 1% agarose gel at 120V for 15 minutes and visualized on a blue light transilluminator [14].

Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer. The sequencing was done at a final volume of 10µl, the components included 0.25ul BigDye terminator v1.1/3.1, 2.25µl 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 96^0C for 10seconds, 55^0C for 5 seconds and 60^0C for 4 minutes [14]

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 BLAST. These sequences were aligned using Clustal X. the evolutionary history was inferred using the Neighbor- Joining method in MEGA 11. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes- Cantor model [14].

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*., 2015 [15]. 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

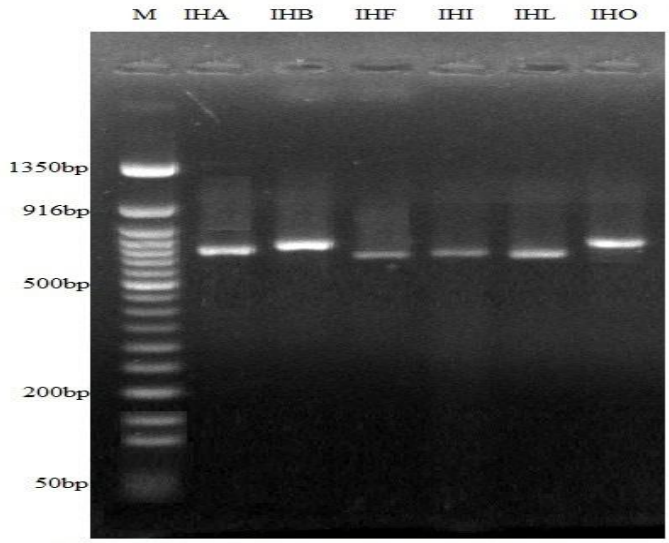
All *Aspergillus* species isolated from the samples were subjected to in-vitro antifungal susceptibility test by Kirby-Bauer disc diffusion method [16].Stock inocula were prepared by adding 24 hours old cultures to 5mL sterile normal saline. The culture was adjusted to a 0.5 McFarland's standard to obtain final inoculum volume of 1.0×10^5SFU/ml.Afterwards, 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 susceptibletothe antifungal agents.

3.0 Results

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 to have a very close relatedness with*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).



Gel image showing the amplification of the Internal Transcribed Spacer (ITS) at 650bp. Lane M is 50bp DNA ladder

Plate.1: Agarose gel electrophoresis output of the amplicons

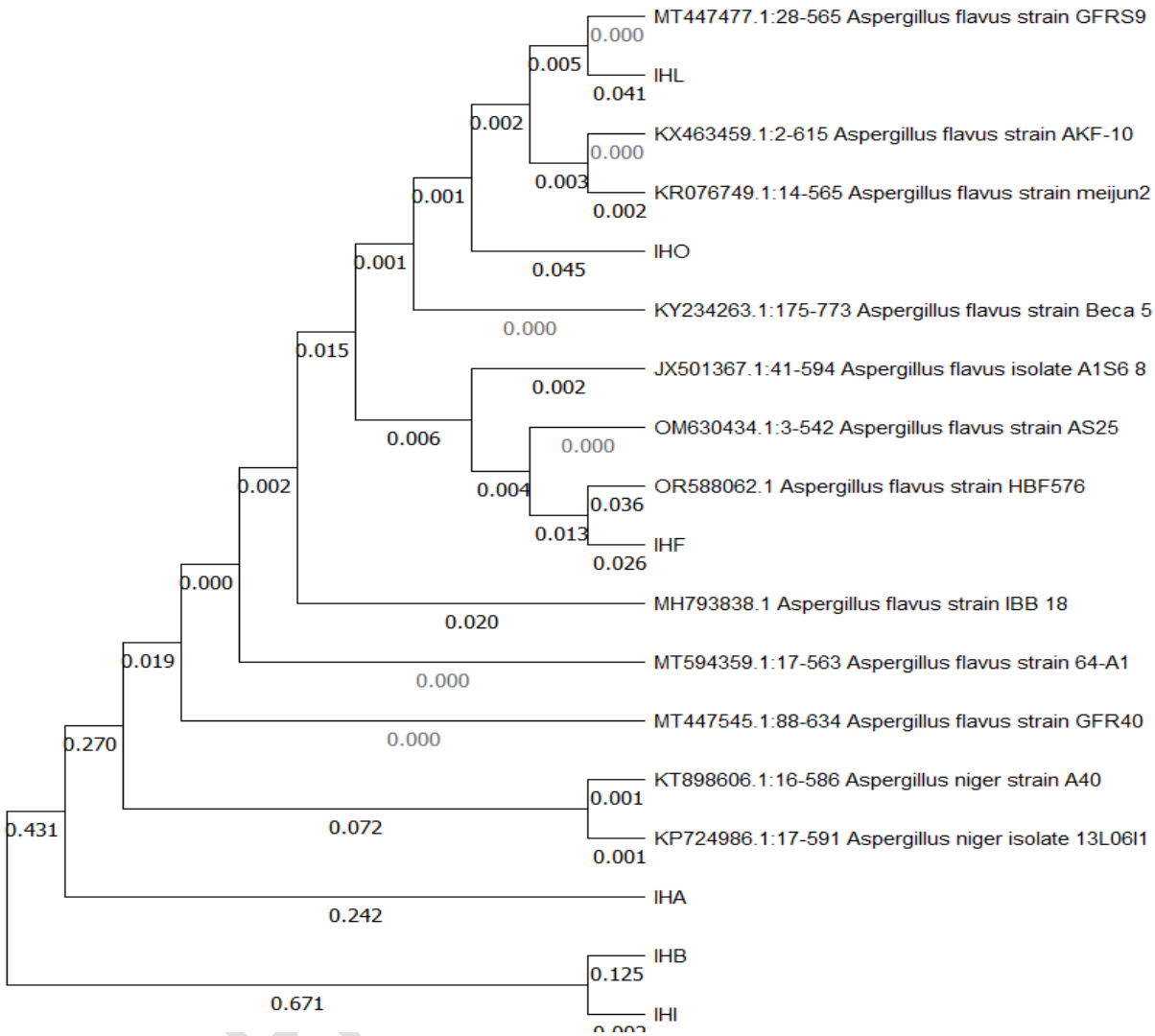
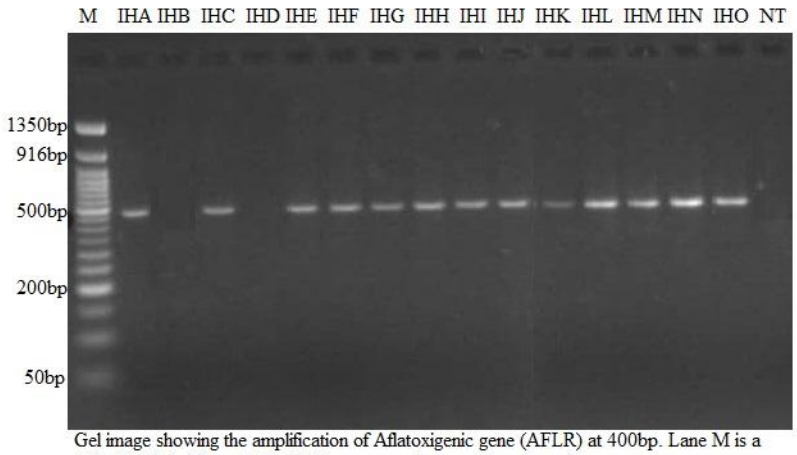


Fig. 1:Evolutionary Relatednessof the Fungal Isolates

Results of the agarose gel electrophoresis for aflatoxigenic gene (Aflatoxin regulatory gene) markers in Plate 2showed that thirteen (86.7%) of the fungal isolates had the aflatoxin regulatory gene while two (13.3%) did not.



Gel image showing the amplification of Aflatoxigenic gene (AFLR) at 400bp. Lane M is a 50bp DNA ladder and lane NT is a no template negative control.

Plate 2:Aflatoxin gene markers of the fungal species.

3.2 Antifungal Susceptibility of the isolates

The results of the response of the isolates to the various antifungal drugs is presented in Table 1. 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, thepercentage(%) susceptibility of the fungal isolates to nystatin was 100% while the susceptibility to fluconazole was 2.86%.

Table 1: Response of the fungal isolates to the conventional antimycotic drugs used for *A. flavus* and *A. niger*

	A. flavus n =22		*A. niger* 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 inthemarkets sampled, were *A. flavus*followed by *A. niger*. The fungal isolates in the present study have been reported to be associated with grains. Krnjaja *et al.*, 2017[17] reported the presence of *Fusarium, Aspergillus* and *Penicillium* sp in their study. The present study however, is in agreement with findings ofShamsuddeen *et al*., 2017[18] who also isolated *A. flavus* and *A. niger* amongst other fungal isolates in grains.

One of the most important mycotoxins that accompany different types of diseases including cancers is aflatoxins [19]. In the present study, aflatoxin gene markers were detected in some of the isolates.The prevalence of the fungal isolates bearing the aflatoxin regulatory gene (aflR) was 86.7% while it was not detected in 13.3% of the isolates. In addition, *A. niger* isolates IHB showed a negative result for the aflRgene while four of the *A. niger* and eight *A. flavus* isolates were positive for the gene. The aflR gene regulates the activity of other structural genes in the aflatoxin biosynthetic 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 pathway [20]. 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 [21, 22].Amaike and Keller, 2011[22] 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.*,2018[23]reported 79% prevalence of aflR gene in 31 strains of *A. flavus* while in the present study, 86.7% from thirteen out of fifteen isolates was recorded. More so, the presence of the aflR gene in *A. niger* might not necessarily imply that they could all produce aflatoxins [23]. Erami *et al*.,2007[24] and Rashid *et al.*,2008[25] 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 [26].

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 iswell 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) [27]. 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[28] in their study reported that nystatin completely inhibited the growth of *A. niger, A. fumigatus,* and *Ochracious* sp 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 [29].

4.0 Conclusion

The species of fungi such as *A. flavus*and *A. niger*identified in the present study could be a public health threat especially as some of the strains *A. niger* and *A. flavus* have been reported to be associated with diseasessuch as cancer and immune deficiencysince they could produce 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.*

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