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Molecular detection of aflatoxigenic *A. flavus* in chicken liver with a special reference to aflatoxin B1

Hams M.A. Mohamed[1*], Asmaa G. Mubarak[2], Fatma A. Khalifa[3]

[1]*Department of Microbiology, Faculty of Veterinary Medicine, South Valley University, Qena, 83523, Egypt.*
[2]*Department of Zoonoses, Faculty of Veterinary Medicine, South Valley University, Qena 83523, Egypt.*
[3]*Department of Infectious Diseases, Faculty of Veterinary Medicine, South Valley University, Qena 83523, Egypt.*

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***Correspondence:**

Corresponding author: Hams M.A. Mohamed
E-mail address: hams.mohamed@vet.svu.edu.eg

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ABSTRACT

Aflatoxin-producing *Aspergillus* species are extensively dispersed throughout the environment and have seriously affected human and animal food supplies, posing health dangers and even resulting in death, as a consequence. Our study's goal was to investigate infections of *Aspergillus* flavus and the amount of aflatoxins (AF) in chicken livers where they are metabolized specially AFB1. Sixty-five chicken liver samples were bought from frozen meat shops, supermarkets, and special slaughterhouses in Qena, Egypt. The samples were evaluated traditionally, molecularly, and HPLC analysis was performed to quantify the amount of AFs. In addition, *A. flavus*' susceptibility to amphotericin B and voriconazole was also determined. The findings revealed the presence of different fungal species, in particular, *Aspergillus* species (21.5%). A total percentage of 85.7 of *A. flavus* isolates were classified as low aflatoxin producers, according to HPLC analyses. Furthermore, aflatoxins contaminated 70% of the liver samples from which AFB1 was detected at 60%. Although this measurement was lower than the European limits, Egyptian standards found it unacceptable. Antifungal susceptibility testing revealed that 71.4 and 42.8% of *A. flavus* isolates were resistant to amphotericin B and voriconazole, respectively. These results show the extent of the role of chicken livers in the transmission of aflatoxicosis to humans. Hinting that these samples are dangerous to consumers. Consequently, there is a need to adopt aflatoxin residue monitoring and controls in all poultry meat; this cost-effective and efficient technology looks to be beneficial for better food safety. Or at least, liver from poultry that has been exposed to aflatoxins should not be consumed by humans until be cleared before slaughtering.

Introduction

Mycotoxins, the secondary metabolites of fungi that produce toxins, are unwanted contaminants in food and feed that have a detrimental effect on both animal and human health (Zahoor-ul-Hassan *et al*., 2010) . Mycotoxins affect over 25% of crops (Ogbuewu, 2011). Aflatoxin contamination accounts for nearly all naturally contaminated foods and feeds (Sultana and Hanif, 2009). Aflatoxins (AFs) are difuranocoumarin derivatives with a pentanone ring (in the instance of AFBs) or a lactone ring (in the instance of AFGs) attached to the coumarin nucleus generated by numerous *Aspergillus* species, including *A. flavus*, *A. parasiticus*, and *A. nominus* (Umaya *et al*., 2021).

Four principal toxins are produced by aflatoxigenic fungi despite the presence of more than 20 known aflatoxins; AFB1, AFB2, AFG1, and AFG2. From public health significance, AFB1 is the most common and most dangerous known aflatoxin, being classified as a human carcinogen. Aflatoxin-producing fungi can contaminate crops in the field at harvest and during storage making them one of the most pervasive and hazardous mycotoxins on animal health especially in warm and humid regions of the world and frequently found in hay, soil, and decomposing vegetation (Mahato *et al*., 2019).

Due to the well-established capacity of aflatoxins to cause cancer and other hepatotoxic effects in both people and animals, aflatoxins have attracted a lot of attention in studies. Aflatoxin has other negative consequences on animals, such as slower growth rates and less effective feeding, reduced egg production, and hatchability, and greater disease susceptibility. Possible animal-derived aflatoxin residues in foods meant for human consumption pose public health concerns. Indeed, commercial fowl liver samples with detectable quantities of these residues could be found (Bintvihok, 2001), besides, their presence in meat, milk, and eggs (Kumar *et al*., 2018). The problem is that once these foods are contaminated by aflatoxins, they cannot be eliminated by the normal cooking process.

Biosynthesis of aflatoxins is one of the time-consuming and difficult processes. There are 18 enzymatic steps and at least 25 genes are involved in generating the enzymes and controlling the synthesis process (Yabe and Nakajima, 2004). The ability of *A. flavus* to produce aflatoxins is determined by the genetic variation of the strains in addition to the environmental conditions. Aflatoxins contaminate a wide range of food commodities including poultry and poultry products especially chicken liver which usually shows the highest mycotoxins residues (Abd EL-Tawab *et al*., 2001).

The World Health Organization (WHO) has categorized AFB1 as a class 1 carcinogen, making it the most cancer-causing aflatoxin. It is widely recognized to cause malnutrition, immune system malfunction, growth suppression, and hepatocellular carcinoma (Thrasher and Crawley, 2009). Generally, when AF is consumed by animals and humans, it is quickly absorbed into the portal blood through the digestive system and transported to the liver where it is metabolized. AFB1 is converted in the liver cells into a variety of metabolites, which are believed to be a substantial source of mycotoxin contamination and may be transferred to consumable animal products in the food chain. Aside from food contamination, occupational exposure to AFB1 has also been linked to employees' inhalation of aflatoxins from contaminated foods, particularly AFB1-infected meals, in factories and industries (Cao *et al*., 2022; Pratap *et al*., 2022).

The qualitative and quantitative measurement of AFs has been devel-

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oped using a variety of analytical techniques, including thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and enzyme-linked immunosorbent assay (ELISA). Detecting aflatoxins using HPLC, is highly sensitive and flexible (Lin *et al*., 1998; Hussain, 2011). The increased sensitivity, repeatability, and reliability of molecular-based approaches assaulted those based on culture, but different markers are used (Alameri *et al*., 2023). To date, four distinct approaches have been employed for the detection of isolates producing aflatoxins using PCR assays; the first approach targets the *omt-1, nor-1*, and *ver-1* genes involved in the synthesis process (Färber *et al*., 1997), the second targets the genes *nor-1*, *apa-2*, and *omt-1* (*omt*A) (Shapira *et al*., 1996), the third target the genes *omt-1, nor-1*, and *ver-1* (Färber *et al*., 1997) and the fourth that amplify specific sequences of the genes *afl*RS, *afl*J, and *omt*B (Rahimi *et al*., 2008). These systems have been also used by both real-time PCR (Rt-PCR) and conventional PCR.

Numerous tactics were used to manage food fungal contamination physically and chemically since invasive aspergillosis is difficult to treat. Polyenes, triazoles, echinocandins, and allylamines are four key families of antifungals that are effective against aspergillosis (Garvey and Rowan, 2023). Amphotericin B, one of the most well-known and established polyenes, has a broad spectrum of activity against most of the fungus. While voriconazole belongs to the triazole class and is typically used as first-line and empiric therapy for many invasive and non-invasive instances of aspergillosis (Bassetti *et al*., 2015).

Thus, this research aimed to detect aflatoxin-producing *A. flavus* in naturally contaminated chicken liver samples collected from markets in Qena, Egypt by using HPLC and PCR methods. Furthermore, we evaluated the susceptibility of *A. flavus* to the anti-fungal agents.

Materials and methods

Ethical approval

The study was authorized by the Animal Ethical Committee for Veterinary Research (VM/SVU/23(1)-03) at South Valley University's Faculty of Veterinary Medicine in Qena, Egypt.

Sampling

Sixty five liver samples were collected from frozen meat stores, supermarkets, and special slaughter shops in Qena city. Samples were transferred to the laboratory of the Department of Microbiology for mycological examination and were stored at -20 °C till analysis.

Isolation and Identification

The isolation was carried out in accordance with Aşkun *et al*. (2010) and Al-Niaeem *et al*. (2015) directions. The components of Dicloran Rose-bengal Chloramphenicol Medium (DRBC) were adjusted as suggested (Samson *et al*., 2007). Each sample was thoroughly blended in a high-speed blender; 10 g of the homogenate was mixed with 10 ml of distilled water in a shaker for 30 minutes at 20°C . Then, 0.1 mL of this mixture was plated on the DRBC medium. The plates were incubated for three to seven days at 25°C. Purification of colonies was done on Malt extract agar. Mold colonies in each plate were investigated macro- and microscopically, the identification was done according to the basics of the published reports (Pitt, 1979; Domsch *et al*., 2007; Pitt and Hocking, 2009).

DNA extraction

According to Al-Samarrai and Schmid (2000), the fungal DNA was extracted as following; in a liquid nitrogen-filled Eppendorf tube, 30 mg of freeze-dried mycelium were ground to a fine powder. The ground mycelium was re-suspended and lysed in 500 µl of lysis buffer (1 mmol/l EDTA, 20 mmol/l sodium acetate, 40 mmol/l Tris-acetate, 1% w/v SDS pH 7.8) by pipetting until the viscosity of the suspension was significantly reduced and the formation of froth indicated the detachment of DNA from polysaccharides. 2 l of RNAseA (Sigma, USA) were added to the mixture, which was then left to sit for 5 minutes at 37 °C. With the addition of 165 µl of 5 mol/l NaCl solution, the majority of the polysaccharides, proteins, and cell debris could be precipitated more easily. The mixture was then completed by repeatedly inverting the tube. The suspension was centrifuged at 13000 revolutions per minute for 20 minutes at 4°C, and the supernatant was immediately transferred to a new tube and 400 µl of chloroform and 400 µl of phenol were added. By gently tilting the tube, the mixture was thoroughly blended until it took on a milky appearance. The aqueous phase was extracted with an equivalent volume of chloroform following 20 minutes centrifugation. Next, the suspension underwent the previously mentioned chloroform extraction. In most cases, the DNA was precipitated with 95% ethanol following centrifugation for 10 minutes, yielding a clear aqueous phase. The precipitated DNA was rinsed with 70% ice-cold ethanol three times, dried, and dissolved in 50 ml of TE buffer (10 mmol/l Tris-HCl, 0.1 mmol/l EDTA pH 7.8) before being placed in storage at -20°C.

Amplification and sequencing of universal ITS gene

The following components were used in the PCR reaction: PCR buffer (20 mMTris-HCl, 0.1 mM of each dATP, dGTP, dCTP, and dTTP, 50 mMKCl), 1.5 mM $MgCl_2$, and 0.3 M of each primer; For the PCR process, 1.5 U of DNA polymerase Taq (Gibco, Maryland) and the *ITS1* and *ITS4* primers (5′-TCC GTA GGT GAA CCT GCG G- 3′ and 5′-TCC TCC TCC GCT TAT TGA TAT G- 3′) (White *et al*., 1990) were added to a total reaction volume of 50 µl. In a Robocycler thermocycler, 40 cycles of amplification were carried out after DNA was first initially denaturation at 95°C for 4.5 minutes. Each cycle was made up of three steps: a denaturation phase at 95°C for 30 s, an annealing step at 50°C for 30 s, and an extension step at 72°C for 1 min. The last extension step was performed at 72°C for 3 min. after the last cycle. The products were amplified and kept at 4 degrees Celsius till usage. Sequences were produced using the forward primer *ITS1* and the backward primer *ITS4* for the database creation. For sequencing, an automated DNA sequencer (Applied Biosystems) and the QIAquick PCR purification kit (QIAGEN, Switzerland) were employed.

Phylogenetic Analysis

The acquired sequences were examined with sequences found in GenBank (NCBI). To define intra- and interspecies homologies, a software

Table 1. Primers sequences and amplicon size of target genes.

Target gene	Primers sequences	Amplified segment (bp)	Reference
*afl*D (Nor1)	ACCGCTACGCCGGCACTCTCGGCAC GTTGGCCGCCAGCTTCGACACTCCG	400	Al-Niaeem *et al*. (2015)
*afl*P(omtA)	GGCCCGGTTCCTTGGCTCCTAAGC CGCCCCAGTGAGACCCTTCCTCG	1024	
aflR	AAC CGC ATC CAC AAT CTC AT AGT GCA GTT CGC TCA GAA CA	800	Umaya *et al*. (2021)

program (DNASTAR, Window version 3.12e) evolution program was used. Sequences that have at least one independent confirmation from a different sequence of the same species (i.e., a sequence similarity of above 95%). The phylogenetic tree was created using MEGA version 2.1 and the ITS gene nucleotide sequence on the two randomly chosen *A. flavus* isolates (Kumar *et al*., 2001).

Production of aflatoxin in YES media

A synthetic medium termed Yeast Extract Sucrose Broth (YES) was used to extract the aflatoxin generated by *A. flavus* isolates. Using a hemocytometer, spore suspensions were made and adjusted to have almost $5x10^6$ spores/mL. 50 mL of sterile YES and 1 mL of spore suspension were placed in a flask, which was incubated at 25°C for seven days. Following incubation, the entire combination was blended, and broth and chloroform were then mixed equally in a flask. The mixture was shaken continuously for 24 hours. The mixtures were divided into two layers using a separator funnel: an upper layer had spores and mycelia, and a lower layer that contained chloroform and mycotoxins. Chloroform phase was maintained in a dark, dry bottle after evaporation in a water bath at 50°C (Khaddor *et al*., 2007).

Extraction of aflatoxins from poultry liver samples

Each sample weighed 20 g, and 100 milliliters of chloroform were added. The mixture was well mixed and homogenized for five minutes at a high speed of 16000 rpm. The derivatization residue was produced by washing the chloroform extract with an equal amount of distilled water, letting it dry on anhydrous sodium sulphate, filtering it, allowing it to evaporate, and then drying it in opaque bottles (Zohri and Saber, 1992).

Aflatoxin determination by HPLC

A protocol described by Namjoo *et al*. (2016) was followed to measure the levels of aflatoxin using high-performance liquid chromatography (HPLC) and a fluorescence detector (Knaur, Germany). This was accomplished by placing toxin extract into the Agilent C18 reversed-phase columns (4.6 mm x 250 mm i.d., 3.5 m), and washing the columns with water, methanol, and acetonitrile (60:30:10) at a flow rate of 1 ml/min. The fluorescence detector was calibrated using the wavelengths of 365 and 440 nm (Excitation/Emission).

Amplification of toxigenic genes

The three genes from the clustered pathway of aflatoxin synthesis are each amplified separately after optimization (Yabe and Nakajima, 2004). The PCR mixture was eventually generated in a 25-µl reaction that contained 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer at a concentration of 20 pmol, 5.5 µl of water, and 5 µl of DNA template. Under the following cycling conditions, the *afl*D gene was amplified: After five minutes at 94 degrees Celsius, there will be 35 cycles of 30 seconds at 67 degrees, 40 seconds at 72 degrees, and 45 seconds at 72 degrees, for a total of ten minutes. For *afl*P: 5 minutes at 94°C, followed by 35 cycles of 30 seconds each at 94°C, 61°C, 72°C, and 10 minutes at 72°C. For*afl*R: 94°C for five minutes, followed by 35 cycles of 94°C for thirty seconds, 50°C for forty seconds, and 72°C for forty-five seconds, then ten minutes at 72°C. The PCR products were electrophoresed on 1.5% agarose gel using a gel documentation system, stained with Ethedium bromide, and seen under ultraviolet light (Alpha Innotech, Biometra).

Antifungal susceptibility of A. flavus

On Muller Hinton Agar (MHA), antifungal susceptibility by disc diffusion was carried out in accordance with the instructions provided in the CLSI M 51-A (CLSI, 2015) Briefly, the entire surface of the MHA was inoculated by applying the undiluted mold stock inoculum suspension to a cotton swab. Amphotericin B (10 g) and Voriconazole (1 g, Oxoid), discs were placed to the surface of each inoculated MHA plate. After 24 and 48 hours at 25-30°C, the plates were read. CLSI M 51-A was used to interpret zone diameters (CLSI, 2010).

Statistical analysis

SPSS version 28 was used to do data statistics. By using a one-way ANOVA, the control mean, standard error, and differences between means were calculated.

Results

Sixty-five samples of the chicken liver revealed 38 distinctive fungus species belonging to nine genera, with the acquisition of the genus *Aspergillus* (21.54%), which included mostly *A. flavus* (10.7%) and *A. fumigatus* (7.6%), as well as other *Aspergillus* species such as *A. terreus* and *A. niger* (1.5% each). *Penicillium* species were the second most common (15.38%). *Candida* and *Rhodotorula* yeast species were also revealed. Other fungus species discovered in liver samples were Fusarium, *Alternaria*, and *Mucor* (Table 2).

Table 2. Occurrence of fungus species in the examined chicken livers samples.

Fungal species (No. of examined samples=65)	No. (%)
Aspergillus species	14 (21.54%)
A. flavus	7(10.7)
A. fumigatus	5(7.6)
A. terreus	1(1.5)
A. niger	1(1.5)
Penicillium species	10 (15.38)
Cladosporium uredinicola	1 (1.54)
Fusarium solani	2(3.08)
Alternaria species	1(1.54)
Mucor species	1(1.54)
Candida species	4(6.15)
Rhodotorula species	2 (3.08)
Scopulariopsis species	3 (4.62)
Total	38(58.46%)

Table 3. Total Aflatoxin levels extracted from *A. flavus* by HPLC method.

Source of toxin	Types of Aflatoxin	No. of positive isolates	Range (µg/L)
A. flavus isolates(no=7)	B1	6	Low producer (2.76-67.49)
	B2	4	Low producer (0.33-2.70)
	G1	2	Low producer (0.10)
	G2	3	Low producer (0.10-1.33)
Total aflatoxin producing isolates		6(85.7%)	0.10-67.49

Two of the seven *A. flavus* isolates (Figure 1) were chosen at random for sequencing analysis to confirm the phenotypic identification. Our isolates were identified as belonging to one species (*A. flavus*) after blasting the amplified section of the ITS gene sequence on Genbank. MegAlign from Lasergene was used to generate phylogenetic trees using the neighbor-joining technique (version7). Two isolates from chicken livers emerged in phylogenetic trees that were almost identical (Figure 2). Our isolates and those listed in GenBank were analyzed to see what similarities and differences there were. Our *A. flavus* isolates (OQ255946 and OQ255945) grouped with reference *A. flavus* strains on Genbank KT983253 and MZ357890 with 100% identity, respectively.

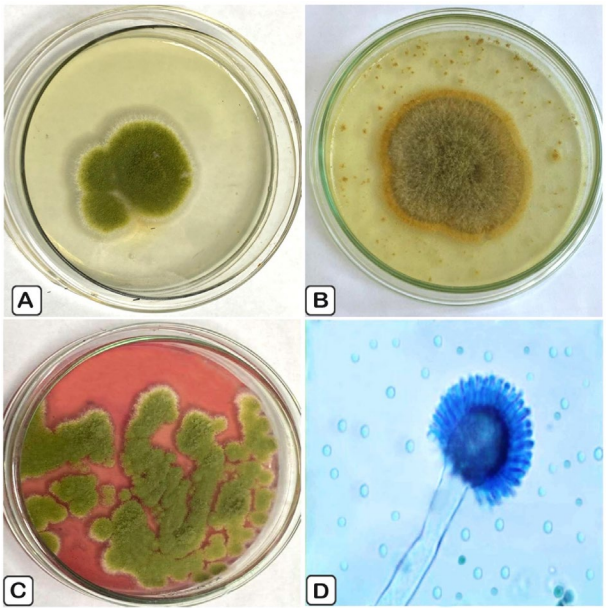

Fig. 1. Macroscopical and microscopical characters of *A. flavus*, *A. flavus* cultivated on SDA, PDA and DRBC (a,b,c).(d) *A. flavus* stained by lactophenol blue under 400X magnification

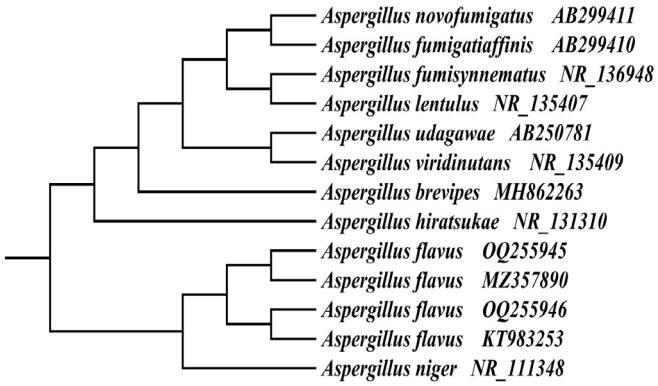

Fig. 2. Phylogenetic tree shows relationships between amplified *A. flavus* OQ255946, OQ255945 ITS region and reference strain registered on GenBank using neighbor-joining method.

To study the aflatoxicogenicity of the isolated *Aspergillus* species, seven isolates were subjected to the quantitative assessment of aflatoxins using High performance liquid chromatography (HPLC). According to the findings, six of the seven *A. flavus* isolates produced various types of aflatoxin at low levels ranging from 0.10-67.49µg/L. All six isolates were found to produce aflatoxin B1, four of them produced B2, two produced G1 and three produced G2 (Table 3). Furthermore, ten liver samples were chosen at random and examined to discover that seven of them had low percentages of aflatoxins (0.08 to 5.27 µg/g), most of them (60%) contaminated with aflatoxin B1 (0.21-5.27 µg/kg) (Table 4).

Additionally, using three sets of primers, PCR was used to examine *A. flavus* isolates for the presence of the *afl*P, *afl*D, and *afl*R genes for aflatoxin B1 (Figure 3 a, b, c). The results of revealed that there is one isolate that harbored only *afl*R gene only, and six isolates had all three of the targeted genes. By comparing the HPLC and PCR data, we discovered that 6 isolates harboured the genes *afl*D, *afl*P, and *afl*R were produced aflatoxin B1, whereas the isolates harboring the aflR gene alone didn't produced aflatoxin B1.

The findings of the antifungal susceptibility test showed that 57.2% of the *A. flavus* isolates exhibited a definite intermediate sensitivity to voriconazole and that 28.5% of the isolates were sensitive to amphotericin B. While 42.8% of the isolates were resistant to voriconazole and 71.5% to amphotericin B (Figure 4).

Table 4. Levels of Aflatoxins naturally occur in chicken livers.

Source of gliotoxin	Types of Aflatoxin	Contamination frequency	Range (µg/kg)
Feed samples (n.=10)	B1	6	0.21-5.27
	B2	2	0.08-0.22
	G1	3	0.21-3.40
	G2	2	0.10-0.18
Total		7 (70%)	0.08-5.27

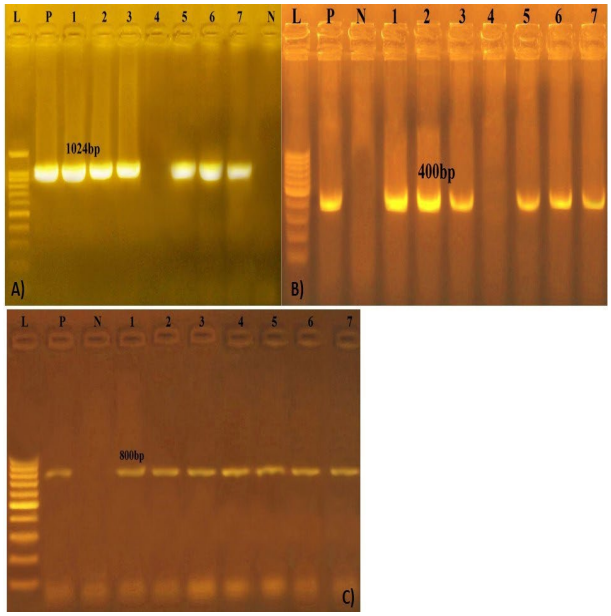

Fig. 3. Agrose gel electrophoresis for amplified *afl*P, *afl*D and *afl*R in *A. flavus* isolates. *A. flavus* isolates lane (1-7), Lane (L): Ladder100bp, lane (P): positive control, lane (N): negative control. (a) *afl*P was amplified at 1024bp, lane:1,2,3,5,6and7 were positive, lane 4 was negative. (b) *afl*D was amplified at 400bp, lane:1,2,3,5,6and7 were positive,lane4 was negative.(c) aflR was amplified at 800bp lane:1,2,3,4,5,6 and 7 were positive.

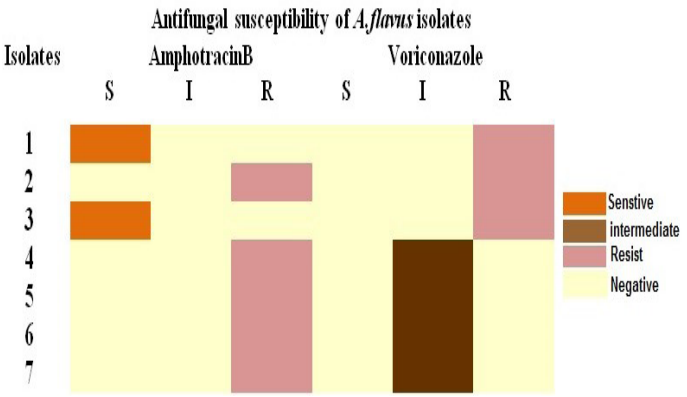

Fig. 4. Heat map of antifungal susceptibility of *A. flavus* isolates.

Discussion

Food microbiologists around the world are becoming more and more interested in fungal contamination of foods, as a cause of food de-

terioration. Numerous research have been conducted to look into how to allay customer concerns about various food safety issues because fungal conidia are already by far the greatest crop destroyer, can move great distances on the wind, and are exceedingly dangerous (Udagawa, 1979)

Fungi are a crucial part of the environment's microflora and are present in a range of substrates in poultry facilities, such as litter, water, manure, and decomposing rodent organic matter. In chicken facilities, fungi and bacteria can thrive easily because of the humidity and temperature that are often constant (Gomes *et al*., 2002; Maciorowski *et al*., 2007). In our investigation, liver samples were found to contain more than thirty fungal species from different genera, similar findings were reported before (Pitt, 1979; Al-Samarrai and Schmid, 2000; Domsch *et al*., 2007; Wadud *et al*., 2012) or on the comb of the chicken (Gründer *et al*., 2005). As well, *Penicillium*, *Fusarium*, *Aspergillus*, *Mucor*, and *Rhyzopus* were the most frequently five fungal species isolated in poultry farms.

Aspergillus and *Penicillium* species are the most commonly found in our investigation which is consistent with the findings of Viegas *et al*. (2012) who discovered the same species in both new and old litter. Although fungus have been demonstrated to interact with the host, they may simply pass through the gastrointestinal tract (Gao *et al*., 2009). One of the most often isolated taxa from soil, decaying plants, and air is *Aspergillus*, which infects people and especially those with impaired immune systems with aspergillosis (Heitman, 2011; Mousavi *et al*., 2016).

In particular, *A. flavus*, which was the most often recovered species from our liver samples was comparable to that reported before (Byrd *et al*., 2017). The identification of fungal species based on morphological characteristics has been supported by numerous studies to explain the evolution of morphological features; however, convergent evolution of fungi has resulted in some cases where traditional methods for categorization of fungi are inaccurate (Hughes *et al*., 2013; Wang *et al*., 2016) so, both traditional and molecular techniques were used to identify *A. flavus* in this study.

For these reasons, it was decided to analyze the evolutionary relationships and confirm phenotypic identification by amplifying the ITS (Internal Transcribed Spacer) region of rRNA genes using *ITS*1 and *ITS*4 primers (Hibbett and Taylor, 2013).The ITS region is frequently utilized as a marker in phylogenetic studies because it contains variable components that allow *Aspergillus* species to be identified using sequences (González-Jartín *et al*., 2022).

The phylogenetic analysis revealed that our isolates shared 100% similarity with *A. flavus* strains in Genbank that were isolated from chicken feed and fowl lung. Consequently, it seems doubtful that the contamination was caused by the dissemination of *A. flavus* spores from an infected lung or that the bird ingested. This hypothesis is backed by Fouad *et al*. (2019), who found that AFB1 is transferred from poultry feed to eggs, meat, and other edible products, posing a risk to consumers' health.

The *A. flavus* and its related aflatoxins, which are among the most prevalent and severe fungal poisons troubling poultry producers, pose a substantial threat to the chicken industry. B1 is the most dangerous and common active toxin among the several forms of aflatoxins, which are classified as B1, B2, G1, and G2. Simply put, aflatoxins affect not just poultry but also other animals and humans (Benkerroum, 2020). According to our findings, aflatoxin production is strictly regulated by *A. flavus*'s growing circumstances. Temperatures between 25 and 30 degrees Celsius are required for considerable amounts of aflatoxin formation in a medium containing readily metabolizable carbohydrates like sucrose. Because YES broth provides all the components necessary for improved toxin production, it was used to culture fungal isolates from chicken livers at 25°C (Klich *et al*.,2009).

When we examined the extract of *A. flavus* isolates by HPLC, 85.7% of *A. flavus* isolates consistently produced low levels (0.10-67.49 µg/L) of the several forms of aflatoxins B1, B2, G1, and G2, The results obtained are supported by Klich *et al*. (2009), who showed that *A. flavus* isolates produced aflatoxin at a low level of less than 100 µg/L. According to Khan *et al*. (2011), two of the *A. flavus* isolates produced aflatoxin at a moderately high level and two at a low level, accounting for 60% of the isolates. Numerous authors have focused on how *A. flavus* produces various kinds of aflatoxin in poultry feed and meat (Anjum *et al*., 2011; Khan *et al*., 2011; Kumar *et al*., 2017).

Aflatoxin in poultry meat was classified as a major concern by the European Union's Rapid Alert System for Food and Feed (RASFF) in 2008 (EC, 2009) due to the serious health effects, and AFB1 was later classified as a group I human carcinogen by the International Agency for Research on Cancer (IARC) (Bernardo *et al*., 2003; Min *et al*., 2011). Aflatoxin still poses a severe threat to food and agricultural goods despite extensive research and management strategies. Aflatoxins in particular assault the liver, causing hepatotoxicity symptoms including fever, malaise, and anorexia, which are followed by stomach pain, vomiting, and hepatitis.

Our findings depicted that the levels of aflatoxins in liver samples that naturally occurred were lower than the restrictions when compared to European standards (EC, 2010), Limits of 8 and 15 µg/kg for total AFs and AFB1 have been determined. The ICMSF (1978) and Egyptian standards (EOS, 2005) stated that the poultry meat must be free of fungal development and associated mycotoxins, while 70% (7/10) of the tested samples were ruled unsatisfactory. However, major mycotoxins in products and food are regulated in at least 100 countries, a significant portion of which focus on aflatoxins. The maximum allowed concentrations differ greatly across countries. In other countries, including Poland and Romania, maximum readings were between 0 and 1 parts per billion, whereas in India, the upper limit was 30 ppb. Therefore, some nations regarded our outcomes as being within their limitations, while others thought they were above their limitations (Agag, 2004).

The evolution of cytochrome P450 isozymes in poultry allows them to convert AFB1 into the more deadly form, AFB1-8,9-epoxide (AFBO), which is why AFB1 is the variation that poses the greatest risk to poultry. This alters the amounts of the AFBO-DNA adduct (which damages DNA) and ultimately affects the synthesis and concentration of AFBO. It's unclear if the same process that makes different poultry species susceptible to AFB1 also explains why a particular fungal strain can create AFB1 that can have detrimental consequences, even at low levels (Klich *et al*., 2009; Gomes *et al*., 2022). Feed contamination is the main cause of mycotoxin infection in animal production, as stated by Diaz *et al*. (2008). AFB1, AFB2, and aflatoxicol have been discovered in the liver, kidneys, and thigh muscles of chicken fed AFB1 with contaminated feed (Razzaghi-Abyaneh, 2013), this is thought to pose a significant risk to the public's health because repeated ingestion of subacute levels of mycotoxins may result in the development of tumors or organ damage (Varshney *et al*., 1991; Girardin, 1997; Forner *et al*., 2015; IARC, 2019).

PCR was an innovative and sensitive method for the early identification of aflatoxin contamination with numerous aflatoxigenic *Aspergillus* species, particularly Aflatoxin B1 (Scherm *et al*., 2005; Davari *et al*., 2015; Bintvihok *et al*., 2016). In the current study, we focus on three attainable genes (*afl*D, *afl*P, and *afl*R) for their primary contribution to aflatoxin B1 production among the many genes implicated in aflatoxin B1 biosynthesis. aflD plays an important role in the early conversion of nosolorinic acid into averantin. On the other hand, *afl*P is involved in converting strigmatocystin into aflatoxin during the latter phases. Additionally, the aflR gene is essential for regulating the expression of other genes involved in the production of aflatoxin (Liu and Chu, 1998; Abdel-Hadi *et al*., 2010; Baquiao *et al*., 2016).

Here, the presence of the three potential aflatoxin B1 genes (*afl*D, *afl*P, and aflR) and the HPLC results for the isolates that produced aflatoxin B1 were exactly correlated. One isolate was discovered to be categorized by HPLC as not producing aflatoxin. However, the *afl*R gene test result was positive. These could be caused by the fact that some *A. flavus* strains that are not aflatoxins producers and provide negative HPLC findings may express at least one of the *afl*P, *afl*D, or *afl*R genes (Davari *et al*., 2015). Furthermore, Dehghan *et al*. (2008) found that numerous other genes and factors account for the aflatoxigenicity of *A. flavus* and that the presence of the *afl*R gene is not necessary for aflatoxin secretion. Environmental factors have a complex influence on the management of aflatoxin, as Bernaldez *et al*. (2017) revealed. In accordance with past studies, the absence of aflatoxin production in some aflatoxigenic species may be due to a simple mutation or deletion of the *afl*R, *afl*D, and *afl*P genes or the loss of additional crucial genes in the aflatoxin production pathway. But different physiological factors might have an effect on aflatoxin formation (Yu, 2012; Baquiao *et al*., 2016).

Aspergillus infection is challenging to treat because of the difficulty in diagnosis, the severity of the clinical condition, and the scarcity of antifungal medications (Amanati *et al*., 2020). In the current investigation, substantial resistance to amphotericin B (71.8%) was seen; our findings were similar to those of Hassan *et al*. (2018) who discovered that 64.4% of *A. flavus* isolates were resistant to amphotericin B. Espinel-Ingroff *et al*. (2011) explained the cause of *A. flavus* resistance to amphotericin B, which is that persistent usage of amphotericin B and its lipid formulations increase selection pressure, making monitoring of emergent polyene resistance in *Aspergillus* spp is critical. Antifungal drug resistance is a growing global issue, both spatially and temporally (Fisher *et al*., 2018), also, Fungal pathogens have been added to CDC (2019), which included a list of urgent antimicrobial resistance (AMR) threats, indicating a rising public health burden.

Conclusion

Serious problems that need to be resolved include fungal infections and the related mycotoxins. In order to distinguish between toxicogenic and non-toxicogenic strains of aflatoxin poisoning in poultry and make an effective treatment and management decision, it may be necessary to identify aflatoxigenic genes or other potential genes in the pathway that leads to aflatoxin production. Additionally, organizations with rules and

regulations pertaining to aflatoxins, such as those in the fields of medicine or veterinary care, could benefit from our research.

Conflict of interest

The authors declare that they have no conflict of interest.

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