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GENOTYPIC CHARACTERIZATION OF SOME PATHOGENIC FUNGI ISOLATED FROM POULTRY AND THEIR SURROUNDINGS

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Abstract: Regarding high morbidity, mortality, and production losses, fungi infections have their importance among infectious illnesses and seem to be one of the main challenges facing poultry producers. This study aims to identify the genotypic characteristics of some fungi isolated from poultry. To reach this end, in El-Gharbia Governorate, Egypt, a total of 210 birds with a history of respiratory distress were randomly selected from a variety of private farms and hatcheries. The birds were sacrificed; tissue pieces were collected. In addition, a total of 87 samples of the poultry surroundings including 40 samples of poultry ration, 14 bedding materials, 4 air samples, and 29 water samples were collected. Using traditional fungal isolation, four fungal species were recovered, namely, *Aspergillus niger, Aspergillus flavus, Cladosporium perangustum,* and *Penicillium chrysogenum*. PCR was performed by fungus-specific universal primer pairs (ITS1 and ITS4) to identify and describe the genotype of isolated fungi. All examined isolates' ITS1-5.8SrDNA regions could be amplified. A purified PCR product was sequenced according to the Emerald Amp GT PCR master mix. This was initially performed to establish sequence identity to GenBank accession numbers. The rRNA gene for 5.8 sRNA divides the two ITS sections, which are situated between the 18S and 28S rRNA genes. ITS-1 gene sequence of the isolated *Cladosporium perangustum* (GeneBank accession number was OM 407392). The Sequence of the ITS-1 of isolated *Penicillium chrysogenum* (GeneBank accession numbers for studied nucleotide sequences were OM407401; OM407402; OM403685, and OM403686). For the examined nucleotide sequences, the GeneBank accession number for the ITS-1 internal transcribed spacer region of single *Aspergillus niger* was OM407391. GeneBank accession numbers for the isolated *Aspergillus flavus* ITS-1 sequence examined nucleotide sequences were OM403676, OM403677, and OM403678. In conclusion, genotypic characterization confirmed the phenotypic traditional fungal identification in the present study. Aspergillus species are the major fungi associated with birds in Egypt farms. The predominantly identified species were *Aspergillus flavus* and *Penicillium chrysogenum.*

Key words: phenotypic; genotypic; molecular; fungi; poultry

Introduction

Poultry is one of the fastest-growing elements of Egypt's agricultural industry, providing income to both skilled and unskilled laborers. It also provides an extra source of income as well as an affordable and accessible supply of protein for several poor households (1). However, lack of knowledge and skills, insufficient finance at all levels, and marketing are all challenges in chicken production (2). Chickens are susceptible to a variety of severe infections that reduce productivity and affect welfare, resulting in significant mortality in some situations. Regarding high morbidity, mortality, and production losses, fungi have their importance among infectious illnesses and appear to be a significant barrier for chicken producers (3). Many fungal infections affect poultry production, including Aspergillosis, Candidiasis, Dactylariosis, Favus, Mucormycosis, Histoplasmosis, and Cryptococcosis. Aspergillosis and Candidiasis are the most common fungal illnesses, although

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Cryptococcosis and Histoplasmosis are also zoonotic (4).

Fungi cause disease in two ways: i) damaging the host's bodily tissues and infiltrating them, and ii) creating mycotoxin during harvesting and storage of crops, which results in illness, immunosuppression, and decreased production potential when ingested (5).

Infections with fungi are frequently linked to morbidity and mortality in birds. Even when the best conditions of culture, harvest, storage, and handling were used, fungi and their toxins are inherent pollutants of the environment, particularly foods. The introduction of outside air containing conidia, which may readily fulfill the nutritional requirements of many fungal species, including Aspergillus species, is another possible source of contamination (6). The majority of fungal diseases in poultry are manifested as high morbidity, mortality, and production on a sporadic basis, but they can also be manifested as an outbreak (5).

The fungal infection primarily affects poultry's respiratory and neurological systems, causing the host to experience pathological changes including inflammation, lesions, and illness that ultimately result in death (3). Risk factors that enhance the possibility of acquiring a fungus and making the situation worse include stress, immunosuppressive sickness, insufficient vitamin D, poor hygiene, chronic use of drugs that impede natural bacterial flora, and malnutrition (7).

The most common fungal illnesses in poultry are aspergillosis and candidiasis (8). Because it causes economic losses in the poultry business, aspergillosis has arisen as a major poultry health concern for poultry producers and human health regulators. Aspergillosis is an infectious, non-contagious disease affecting human beings, mammals, and primarily wild or domestic birds. The most harmful fungus that affects poultry, *Aspergillus fumigatus*, causes aspergillosis, a disease of lung with hematogenous dissemination that causes necrosis and granulomatous cavities (9). Due to the lower spore size compared to other Aspergillus species, Aspergillus fumigatus infection in poultry is more prevalent. *A. flavus, A. terreus, A. glaucus, A. nidulans, and A. niger* are other Aspergillus species that can harm birds (10).

Traditional microscopic and cultural features are still often utilized to identify fungi. Conidial and mycelial color, colony reverse color, colony diameter, soluble pigments, and exudate production are used to make macro-morphological identification. Microscopically, seriation, vesicle size and form, conidia, and stipe morphology all play major roles in fungal identification **(**11). However, these approaches are time-consuming, involve extensive technological skill, and are occasionally ineffective due to the unusual characteristics of isolates. Hence, it is now possible to identify fungi more quickly and accurately thanks to molecular methods (12).

PCR allows the rapid detection of fungal species directly from clinical specimens with good specificity, DNA barcoding with dual loci (ITS) offer optimal accuracy and next generation sequencing technologies offer highly discriminatory analysis of genetic diversity including outbreak investigation, and for drug resistance characterization. Advances in molecular technologies will further enhance routine fungal diagnostics (13).

The aim of this study is to identify the genotypic characteristics of some fungi isolated from poultry and their surroundings.

Material and methods

Sample collection

In El-Gharbia Governorate, Egypt, a total of 210 birds with a history of respiratory distress were randomly selected from a variety of private farms and hatcheries (180 broiler chicks at age of 1:10 days, 5 Baladi breeds at age of 30:45 days, and 25 Saso chickens at age of 30 days). When compared to other farms between November 2020 and July 2021, these farms displayed a distinct mortality rate. The birds' tissues (lung, air sac, liver, crop, and trachea) were taken from each bird.

In addition, a total of 87 samples of the surroundings of the poultry including (40 samples of poultry ration, 14 bedding materials, 4 air samples and 29 water samples) were collected.

Identification and mycological examination according to previous method (14)

The prepared samples were cultured on to Sabouraud dextrose agar plates with a sterile bent glass rod. The cultured plates were incubated at 25°C and examined daily for five days. Mold colonies were picked up and sub-cultured on slope

agar for further identification. The mold culture was purified by subculturing on Sabouraud agar plates, incubated at 25°C for 3-5 days and examined for macro and micromorphological characters.

Genotypic and molecular characterization

The genotypic characterization was performed at Animal Health Research Institute, Eldokki, Cairo, Egypt.

Extraction of DNA

For identification and genotypic characteristics of the isolated fungi, PCR was carried out using the QIAamp DNeasy Plant Mini kit Catalogue No. 69104, the internal transcribed spacer1 (ITS 1 Oligonucleotide primer sequence**:** TCCGTAGGT-GAACCTGCGG), and (ITS 4 Oligonucleotide primer sequence: TCC TCC GCT TAT TGA TAT GC) according to a previous method (15).

This was performed according to QIAamp DNeasy Plant Mini kit instructions.

Making a traditional PCR Master Mix

The Emerald Amp GT PCR master mix (Takara) Code No. RR310A kit was used to sequence a purified PCR product. This was initially carried by mixing Emerald Amp GT PCR master mix (2x premix) 12.5 *µl*, PCR grade water 5.5 *µl*, Forward primer (20 pmol) 1 *µl* , Reverse primer (20 pmo1) 1*µl* and Template DNA 5 *µl* to verify the sequence's connection to GenBank accessions. **Table (1)** displays the temperature and timing parameters for the two primers during PCR. 100 bp gene ruler DNA ladder from Fermentas (cat. no. SM0243) was used during electrophoresis. During electrophoresis grade agarose (1.5 g) (16) was used after the addition of 0.5 g/ml ethidium bromide. The positive control, the negative control, and 20 µl of each PCR product sample were placed onto the gel. A gel documentation system was utilized to take pictures of the gel, and computer software was used to analyze the results.

Phylogenetic research

The sequences (17) in MEGA (Molecular Evolutionary Genetics Analysis is asoftware enables comparative analysis of molecular sequences in phylogenetics) were compared using the CLUSTAL W multiple sequence alignment technique, version 1.83 of the MegAlign module of Lasergene DNAStar software Pairwise, and phylogenetic analysis was performed using maximum likelihood, neighbor joining, and maximum parsimony (18).

Results

Incidence of molds in poultry samples

From 210 birds with 1050 tissue pieces, 306 fungal spp. were isolated and identified. The highest incidence was in lungs among the broiler (25%) and Baladi (0.9%) birds followed by the air sac (16.44%) then trachea (14.37). In Saso birds, the highest incidence was in air sacs (6.2%) followed by the lungs (5.5%) then the trachea (4.4%).

Mycological examination of 87 surroundings samples revealed the isolation of 19 strains mold spp**.,** the mycotic incidence was the highest in ration (50%) followed by the bedding (28.5%)**.**

The most prevalent fungi isolated are *Aspergillus spp.* especially *Aspergillus flavus,* and *Peniciillium spp.*

Using traditional fungal isolation, four fungal species were recovered, namely, *A. niger, A. flavus, Cladosporium perangustum,* and *Penicillium chrysogenum.* The recovered isolates were further identified, and their genotyping characteristics were determined using PCR and a universal primer unique to fungi. The rRNA gene for 5.8 sRNA divides the two ITS sections, which are situated between the 18S and 28S rRNA genes (Fig. 1-4).

Molecular identification of the isolated molds

Confirmatory identification, phylogenetic analysis, and multiple alignments:

The sequence of the ITS-1 of isolated *Cladosporium perangustum* (ES2: isolated from ration) GenBank accession number for the studied nucleotide sequence was OM 407392. More than 99 percent of the sequences found for the ITS-1 region matched the relevant GenBank sequences (accession no. MT645915; KT600413; KT600414; HM148147 and HM148141) (Fig. 5).

Table 1: Cycling conditions of the different primers during conventional PCR

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
ITS	95˚C 5 min.	94˚C 30 sec.	56˚C 40 sec.	72˚C 50 sec.	35	72˚C 10 min.

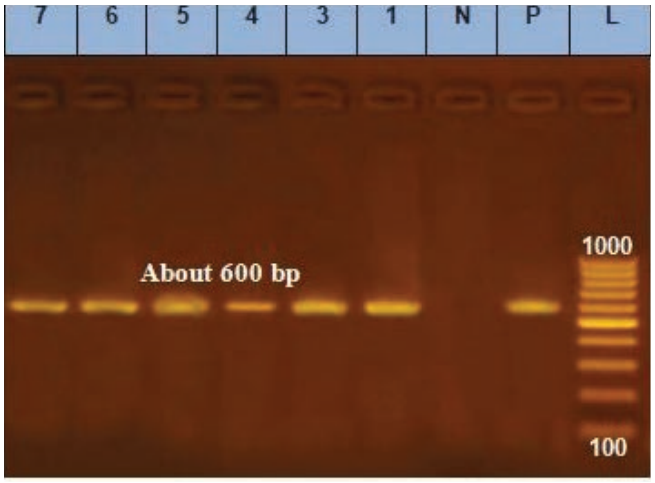

Figure 1: PCR findings for six fungal isolates. Amplification of the 600 bp fragment of ITS1 gene from tested isolates (Lanes 1-7) showed a positive amplicon migration with the molecular size of about 600 bp using the molecular DNA size marker.

L: 100 bp ladder. P: control positive.
N: control negative.

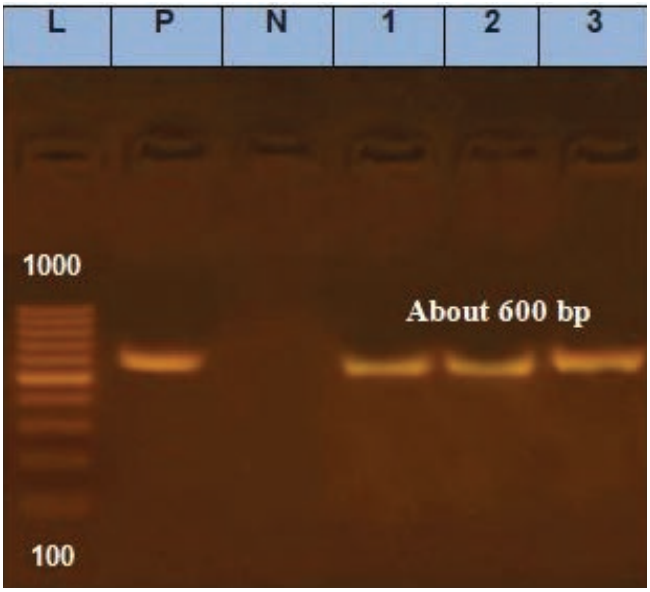

Figure 2: PCR findings for three fungal isolates. Amplification of the 600 bp fragment of ITS1 gene from test-ed isolates (Lanes 1_ 3) showed a positive amplicon migration with the molecular size of about 600 bp using the molecular DNA size marker. L: 100 bp ladder. P: control positive. N: control negative.

The sequence of the ITS-1 of the isolated *Penicillium chrysogenum* (ES6: isolated from bedding materials, ES7: isolated from lung tissue and ES1: isolated from ration) GenBank accession numbers for the studied nucleotide sequences were OM407401; OM407402; OM403685, and OM403686. Over 99 % of the matched GenBank sequences and the ITS-1 region sequences were comparable (accession no. KP27816).

GenBank accession numbers for the examined nucleotide sequences were OM407391 for the isolated *Aspergillus niger* (ES9: isolated from bedding materials and air samples) isolate's internal transcribed spacer-1 region (ITS-1). The sequences obtained for the ITS-1 region obtained were 100% identical to the corresponding GenBank (accession no. MG 228419; MG228418; MG228417; MG228416; MH855726, and MH855928). For the examined nucleotide sequences, the GenBank accession codes for the ITS-1 of isolated *Aspergillus flavus* (ES4: isolated from lung tissue, ES5: isolated from air sac and ES8: isolated from trachea) were OM403676, OM403677, and OM403678. The sequences obtained for the ITS-1 region were 100% identical to the corresponding GenBank (accession no. MT292809; CP051089; MT462229; MT629885; MN095128, and MH864265).

Discussion

The present study was conducted aiming to the genotypic characterization of some fungi isolated from broiler, Baladi, and Saso chicken from different farms in Egypt.

The most prevalent fungal species isolated was Aspergillus species especially *Aspergillus flavus* and *Penicillium spp.* These findings were in line with a previous report (19), where *Aspergillus spp.*, and *Penicillium spp.*, were the most prevalent fungal species detected in poultry.

Fungal diseases are a severe threat to human and animal health around the world. Infections with fungi are frequently linked to morbidity and mortality in birds. Even when the best conditions of culture, harvest, storage, and handling were

Figure 3: ITS-1 and ITS-2 Nucleotide sequence alignment of *A. flavus; A. niger; P. chrysogenum and C. perangustum* and other intra- and interspecies fungal strains

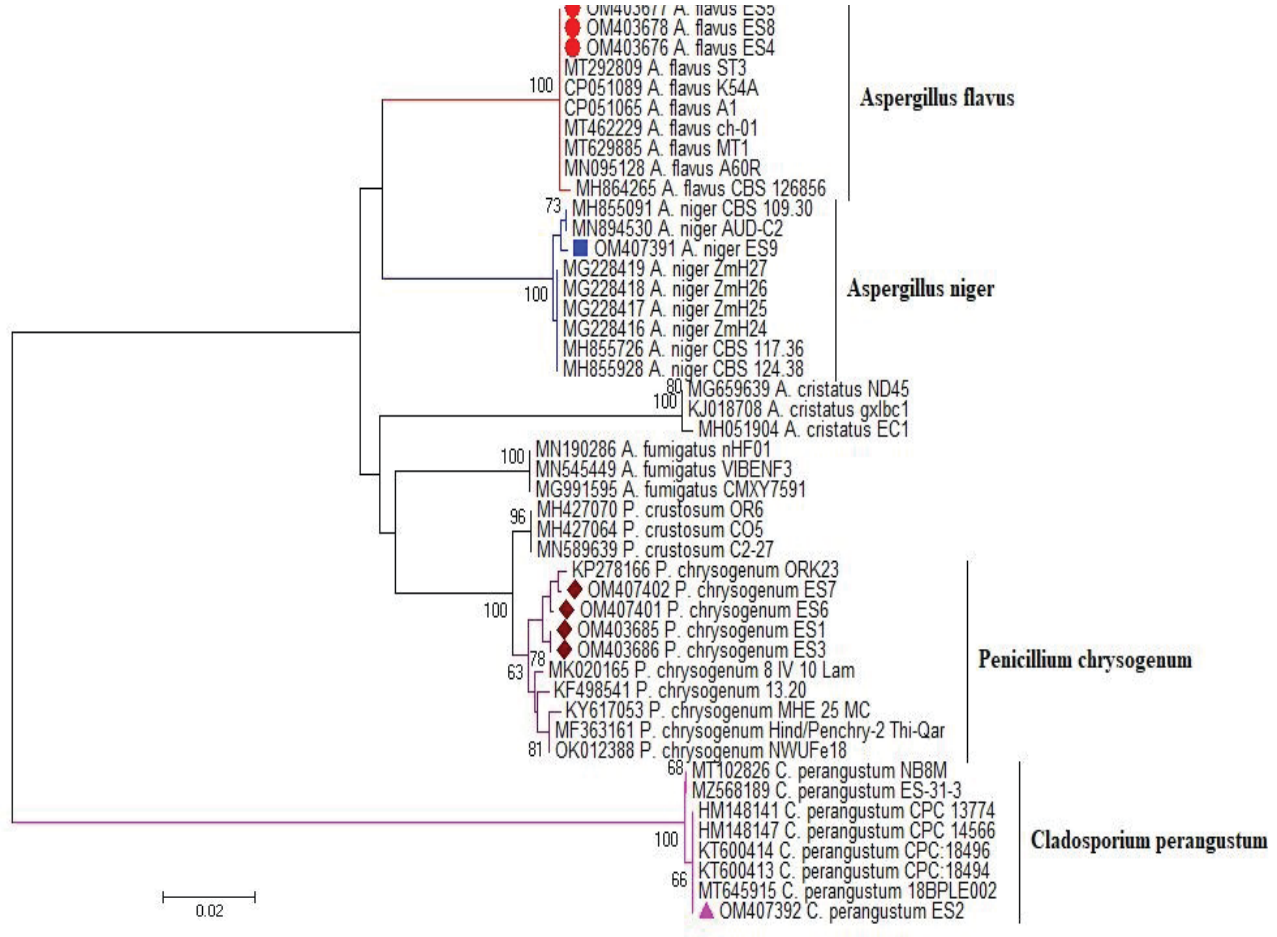

Figure 4: Phylogenetic analysis of strains (ES4, ES5, ES8, ES9, ES1, ES6, ES7, ES3, and ES2) with other intra- and interspecies strains. The tree is showing that the strains are very close to the other stains. Other strains are showing lesser or greater distance, filled squares indicate our isolated strains

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		
1		99.8	99.8	99.8	99.8	85.8	85.8	85.6	64.7	64.9	64.7	85.3	85.8	85.4	86.0	85.8	85.3	76.7	76.9	84.3	84.3	1	MH864265 A. flavus CBS 126856
2	0.2		100.0	100.0	100.0	86.0	86.0	85.8	64.9	65.1	64.9	85.4	86.0	85.6	86.2	86.0	85.4	76.9	77.1	84.5	84.5	2	MT629885 A. flavus MT1
3	0.2	0.0		100.0	100.0	86.0	86.0	85.8	64.9	65.1	64.9	85.4	86.0	85.6	86.2	86.0	85.4	76.9	77.1	84.5	84.5	3	OM403676 A. flavus ES4
4	0.2	0.0	0.0		100.0	86.0	86.0	85.8	64.9	65.1	64.9	85.4	86.0	85.6	86.2	86.0	85.4	76.9	77.1	84.5	84.5	4	OM403677 A. flavus ES5
5	0.2	0.0	0.0	0.0		86.0	86.0	85.8	64.9	65.1	64.9	85.4	86.0	85.6	86.2	86.0	85.4	76.9	77.1	84.5	84.5	5	OM403678 A. flavus ES8
6	11.7	11.4	11.4	11.4	11.4		100.0	99.8	64.0	64.2	64.0	85.6	86.8	85.6	86.0	86.2	85.8	76.5	76.9	88.1	88.1	6	MG228419 A. niger ZmH27
7	11.7	11.4	11.4	11.4	11.4	0.0		99.8	64.0	64.2	64.0	85.6	86.8	85.6	86.0	86.2	85.8	76.5	76.9	88.1	88.1	7	MH855726 A. niger CBS 117.36
8	11.9	11.7	11.7	11.7	11.7	0.2	0.2		63.8	64.0	63.8	85.4	86.6	85.4	85.8	86.0	85.6	76.3	76.7	87.9	87.9	8	OM407391 A. niger ES9
9	37.1	36.7	36.7	36.7	36.7	38.8	38.8	39.2		99.8	100.0	65.3	66.0	65.3	65.7	65.9	65.1	61.4	61.2	64.0	64.0	9	HM148141 C. perangustum CPC 13774
10	37.1	36.7	36.7	36.7	36.7	38.8	38.8	39.2	0.0		99.8	65.5	66.2	65.5	65.9	66.0	65.3	61.6	61.4	64.2	64.2	10	KT600413 C. perangustum CPC:18494
11	37.1	36.7	36.7	36.7	36.7	38.8	38.8	39.2	0.0	0.0		65.3	66.0	65.3	65.7	65.9	65.1	61.4	61.2	64.0	64.0	11	OM407392 C. peranguhstum ES2
12	13.0	12.7	12.7	12.7	12.7	13.4	13.4	13.6	37.5	37.5	37.5		97.4	99.4	99.3	99.4	99.3	77.8	78.2	87.3	87.3	12	KP278166 P. chrysogenum ORK23
13	12.5	12.3	12.3	12.3	12.3	12.6	12.6	12.9	36.5	36.5	36.5	2.2		97.9	98.1	97.9	98.1	79.3	79.7	88.4	88.4	13	KF498541 P. chrysogenum 13.20
14	12.7	12.5	12.5	12.5	12.5	13.4	13.4	13.6	37.5	37.5	37.5	0.6	1.6		99.4	99.3	99.4	78.2	78.5	87.7	87.7	14	OM403685 P. chrysogenum ES1
15	12.2	12.0	12.0	12.0	12.0	13.1	13.1	13.3	37.0	37.0	37.0	0.6	1.6	0.4		99.8	98.9	78.5	78.9	88.1	88.1	15	OM407401 P. chrysogenum ES6
16	12.5	12.2	12.2	12.2	12.2	12.9	12.9	13.1	36.7	36.7	36.7	0.4	1.8	0.6	0.2		98.7	78.4	78.7	87.9	87.9	16	OM407402 P. chrysogenum ES7
17	13.0	12.7	12.7	12.7	12.7	13.1	13.1	13.4	37.9	37.9	37.9	0.8	1.4	0.6	1.0	1.2		78.0	78.4	87.9	87.9	17	OM403686 P. chrysogenum ES3
18	15.9	15.6	15.6	15.6	15.6	15.5	15.5	15.8	37.6	37.6	37.6	14.3	12.5	13.8	13.5	13.7	14.1		99.6	77.8	77.8	18	MH051904 A. cristatus EC1
19	15.8	15.6	15.6	15.6	15.6	15.2	15.2	15.5	38.3	38.3	38.3	14.0	12.2	13.5	13.2	13.4	13.8	0.2		78.2	78.2	19	MG659639 A. cristatus ND45
20	12.3	12.0	12.0	12.0	12.0	9.3	9.3	9.6	37.7	37.7	37.7	9.0	7.9	8.6	8.3	8.6	8.3	13.7	13.4		100.0	20	MN190286 A. fumigatus nHF01
21	12.3	12.0	12.0	12.0	12.0	9.3	9.3	9.6	37.7	37.7	37.7	9.0	7.9	8.6	8.3	8.6	8.3	13.7	13.4	0.0		21	MN545449 A. fumigatus VIBENF3
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		

Figure 5: Sequence distance for confirming the percentage of identity between the isolated strains and other related stains on GenBank and all are identical by more than 99% or 100%

used, fungi and their toxins are inherent pollutants of the environment, particularly foods. Other contamination might include improper bedding management, poor quality feedstuff, or admission of outside air containing conidia that can easily meet the nutrient requirements of many fungal species, including *Aspergillus spp.,* (6).

Rapid and accurate separation of pathogenic *Aspergillus spp.* has become critical for selecting effective antifungal medication. Furthermore, species identification is essential for epidemiological and management purposes, such are the correct assessment of incidence rates, observation of the advent of novel species, and invasive aspergillosis outbreaks (20). Additionally, molecular characterization of *Aspergillus spp.* by PCR and sequencing of PCR products were very important)21). The goal of the current study was to genotypically characterize certain fungi that were isolated from broiler, Baladi, and Saso chicken as well as their surroundings, including their diet, bedding, air, and water samples, from various farms in Egypt.

In the present study, the molecular identification and genotyping characters of the isolated fungi were performed using PCR-based identification techniques. The identification systems for the isolated pathogens were based on ITS 1 and ITS 2 regions with the use of 18S or 28S rDNA as target DNA. This agreed to previous reports)7, 22(.

The fungi's sequenced ITS regions supported the morphological investigations' conclusions. In this investigation, the PCR products of the examined samples were identified as *Cladosporium perangustum, Penicillium chrysogenum, A. niger,* and *A. flavus*. The sequences obtained for the ITS-1 region of the isolated *Cladosporium perangustum*, and the isolated *Penicillium chrysogenum* were 99% identical to the corresponding GenBank records, while for the isolated *A. niger*, and *A. flavus* were 100% identical to the corresponding GenBank records.

It is concluded that *Aspergillus spp.* is associated with poultry birds in Egypt farms. The genotypic characterization of fungi from poultry and the surroundings showed that the predominantly identified species were *A. flavus* and *Penicillium chrysogenum*.

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