Mansoura Veterinary Medical Journal 21:1 (2020) 6-13

https://doi.org/10.35943/mvmj.2020.21.102

Original Article *Bacteriology*

Detection of mold species in poultry farms in refer to their virulence potential

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ARTICLE HISTORY

Received: 02.01.2020

Revised: 30.01.2020

Accepted: 23.03.2020

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ABSTRACT

Objective: The aim of the present study was to isolate and identify mold species from poultry farms with detection of their virulence potential, biofilm formation capability and to perform antifungal susceptibility testing to some representative isolates.
Design: Observational study.
Animals: Fifty, freshly dead broiler chicks.
Procedures: A total of 250 samples were collected from 50 diseased chicks (5 samples each), including lung, liver, kidney, heart, and tracheal swap. In addition, litter samples were collected from 7 poultry farms and were subjected to mycological examination. The isolated mold species have been tested for hemolytic activity, catalase, amylase, lipase, and biofilm production activity; besides, detection of virulence genes (rhbA, fos-1, and pskB) using PCR assay. .
Results: A total of 208 mold isolates were identified, with five genera; Aspergillus (84.6%), Zygomycetes (12.9%), Acremonium (0.96%), Penicillium (0.96%) and Alternaria (0.48%). Mold isolates displayed various degrees of fungal activities on blood agar plates, catalase activity, amylase activity, lipase activity, and the ability for biofilm production in vitro. Regarding the selected virulence genes, fos-1 was detected in *A.fumigatus* (3 isolates) and *A.flavus* (2isolates). While pksP gene was detected in *A.fumigatus* (7 isolates) and *A.niger* (2 isolates) and rhbA detected in *A. fumigatus* (8 isolates) and one isolate of *A. flavus* of the total evaluated species. The MIC determination provide evidence for the high resistance of all evaluated isolates to nystatin, and a relatively higher sensitivity was displayed by clotrimazole followed by ciclopiroxolamine and tioconazole.
Conclusion and clinical relevance: The results reveal that most of the fungal isolates tested displayed enzymatic activity, which are the most effective virulence factors contributing to fungal pathogenicity and high resistance to antifungal, which represents a potential public health concern.

Keywords: Molds, Broiler chicks, Antifungals, Enzymatic activity, Virulence genes.

1. INTRODUCTION

Fungal/mycotic diseases cause significant economic losses to the poultry industry either due to their direct infectious nature or due to the production of mycotoxins. They induce high morbidity and mortality in young birds, stunted growth, and diarrhea. Mycotic infections in poultry are frequently associated with immunosuppression, which predisposes many bacterial and viral infections **[1]**. In addition, some fungi such as *Aspergillus* species have significant public health importance, especially in immunocompromised patients **[2]**.

The source of infection to poultry farms occur either via using of a moldy litter or from hatcheries facilities when one-day-old birds have retained conidia arrived at the farms. Other sources of farm contamination may occur through poor quality feedstuffs, improper bedding management, the entrance of air loaded with conidia **[3, 4]**. In poultry farms, humidity, and temperature conditions stimulate the hyphal growth and multiplication of fungi leading to the huge production of conidia, which are subsequently spread and inhaled by the birds **[5]**.

Virulence factor is a microbial element causing host damages and considered a determinant of pathogenicity **[6]**. Fungi produce an array of extracellular hydrolytic enzymes, such as lipases, proteases, and phospholipases. These enzymes play an essential role in fungal pathogenicity, such as in fungal nutrition, tissue damage, iron acquisition fungal dissemination within the human body, and overcoming the host immune system **[7]**. Fungal hydrolytic enzymes not only facilitate easier tissue invasion but also they could impair

some mechanisms of the immune system and assist in obtaining nutrients, subsequently causing damage to the host **[8]**. Additionally, fungi possessing several genes and proteins associated with their pathogenicity **[9]**, *fos-1* is a gene encoding a putative two-component histidine kinase which responsible for osmotic change adaptation regulation, while, *pksP*, a putative polyketide synthase encoding gene, involved in pigment biosynthesis **[10]**. The expression of *pksP* detected in vitro and in vivo has been previously studied by **Tsai et al. [11]** who found transcripts of *pksP* only during conidiation and not in the hyphal stage by northern blot. The Rheb proteins comprise a family of Ras-related proteins that exhibit deviations from the consensus amino acid sequence in the first GTP-binding domain, as well as in the effector domain **[12]**. In a previous study, **Panepinto et al. [13]** created a strain that lacks *rhbA* gene, the Δ*rhbA* mutant displayed a considerable reduction in virulence compering with the wild virulence type.

Biofilms are defined as a community of microorganisms that are attached to a surface and embedded in an extracellular polysaccharide matrix, which facilitates the adherence of these microorganisms to biomedical surfaces concentrate the enzymes produced during the growth which helps in further tissue invasion and protect them from host immune system and antimicrobial therapy and associated with a variety of chronic and persistent infections. The infections associated with biofilm formation are difficult to be cured with existing drug therapies which leads to a high mortality rate **[14]**.

Antifungal susceptibility tests are very important tools to direct the treatment of fungal diseases, to detect antifungals resistance, and to recognize disease epidemiology **[15]**. The resistance in fungi may be developed due to excessive use of drugs in farm or due to genetic mutation of the drug target in fungi- or due to secretion of the fungal enzyme such as 14- α-sterol demethylase which are responsible for the resistance to azole drugs. In addition, there are other mechanisms of antifungal resistant include increased efflux pump activity and decreased target enzyme affinity, also in vivo, there are mechanisms of antifungal resistance such as biofilm formation, which protects fungi from the action of antifungals **[16-20]**.

Thus, the current study was planned for the mycological examination of diseased chicks as well as poultry litter to recognize the possible occupational exposure of poultry farmworkers to fungi and their metabolites from poultry farms located at Dakahalyia governorate, Egypt with detection of its virulence determinants and to determine its susceptibility to antifungal agents.

2. MATERIALs AND METHODS

2.1. Samples collection

Tissue samples including liver, lungs, heart, kidneys as well as tracheal swab samples were collected from 50 freshly dead chicks collected from seven different chicken poultry farms located in the district of Mansoura City, Egypt, during the period from July to December 2017. On necropsy, the most common lesions detected were congestion in the lung associated with the presence of nodule in lung tissue. In addition, litter samples were collected from the visited farms. Each sample was kept separately in a sterile plastic bag, and the bags were labeled and kept on ice containers and transported to the laboratory for mycological examination.

2.2. Isolation and identification of fungal isolates

From each chicken visceral organ, tissue specimen was cultured after being touched by flamed spatula then homogenized before inoculation. Tracheal swabs were streaked with the cotton swaps and litter samples directly streaked onto SDA (cat. No. CM2497E) and PDA (was made by mixing 200gm potato infusion, agar (15 g) and dextrose (20g) plates supplemented with chloramphenicol to inhibit bacterial growth, each sample was cultured onto two plates to be incubated at 25°C and 37°C and checked daily for any fungal growth until ten days **[21]**. The isolated fungi were identified macroscopically by observing colony characters and colony reverses in the inoculated plates and microscopically using scotch tape preparation stained with lactophenol cotton blue stain **[22]**.

2.3. Blood Hemolysis test:

Determination of hemolysin activity was evaluated with a blood plate assay, according to **Manns et al. [23]** using PDA supplemented with 7% fresh sheep blood and 3% glucose **[24]**.

2.4. Detection of mold enzymatic activity

Amylase production was evaluated using the starch agar plate method, as described by **SB et al. [25]**. In brief, fungal isolates were inoculated into potato dextrose agar supplemented with 1g of starch. Lugol's iodine solution was added to the culture plate to identify the zones around the cultures. Additionally, Catalase activity was assessed by using the H_2O_2 solution, according to **Zohri et al. [26]**. Furthermore, lipolytic activity was determined, as described by **Alapont et al. [27]**. In brief, agar plates containing Tween 80 were prepared. Positive results were observed by the formation of white precipitation along the periphery of the colony.

2.5. Biofilm production

The ability of biofilm formation was determined by tube adherence test, according to **Dag et al. [28],** by using 2% safranin. They were then examined for the presence of an adherent layer. Biofilm production was scored as negative (-), weakly positive (+), or strong (++) positive.

2.6. Determine Minimal inhibitory concentration (MIC)

The activity of fungal isolates was evaluated against clotrimazole, tioconazole, ciclopiroxolamine, fluconazole, and nystatin. A stock solution was prepared from each antifungal drug to get a final concentration of 1000 µg /ml and kept at 4◦C until assayed. MIC for each fungal isolate was evaluated according to the method described by **Ochei et al. [29]** and **Agbulu et al. [30]**.

2.7 PCR amplification and gel electrophoresis

In total, 49 fungal isolates were screened for the presence of three virulence-associated genes. The isolates including, A. *fumigatus* (9 isolates), Zygomycetes (9 isolates), *A.niger* (8 isolates), *A. flavus* (9 isolates), A. *terreus* (5 isolates), *A. glaucus* (3 isolates), *A.* nidulans (one isolate), Acremonium (Two isolates), Penicillium (Two isolates); and Alternaria (Two isolates). Fungal DNA was extracted by the QIAamp DNeasy Plant Mini kit (Catalogue no. M501DP100). PCR was carried for detection of three virulence genes including, *fos-1* encoding a putative two-component histidine kinase, *PksP* encoding Polyketide synthase (DHN-melanin synthesis) and *rhbA* is a Ras-related protein. Primer pairs used **[31]**. (target genes, sequences, and PCR products) are summarized in Table 1. PCR was performed in a volume of 25 µL consisting of 12.5 µL of 2X ABT Red Master Mix (Cat. No ABT003), 1 µL of each diluted primer, and 5 µL DNA template, nuclease-free water was used for complete the volume into 25 µL. PCR program was conducted, as mentioned by the referred authors for each gene (Table 1). The PCR products were fractionated on a 1.2% agarose gel using a 1X TBE buffer containing 3 µL ethidium bromide and using ABT100bp plus DNA ladder (Cat. No ABT011) and were visualized under UV light, and the gels were photographed using a UV gel documentation system.

3. RESULTS

In total, 208 isolates were identified by macroscopic and microscopic examination including, 196 isolates from the diseased birds and 12 isolates from litter samples. Five genera were identified: Aspergillus (84.6%), including six species (*A. fumigatus* (32.69%), *A. flavus* (20.6%), *A. niger* (26.9%), *A. terreus* (2.4%), *A. glaucus* (1.4%), *and A.nidulans* (0.48%), Zygomycetes (12.9%) including Mucor and Rhizopus, Acremonium (0.96%), Penicillium (0.96%) and Alternaria (0.48%) (Table 2).

The distribution of fungal isolates in different samples is presented in Table 2, Nineteen *A. fumigatus* isolates were recovered from the lung tissue. The prevalence of *A. fumigatus* was higher in winter than in hot climate followed by *A. niger* (15 isolates) and *A. flavus* (11 isolates), Zygomycetes (6 isolates), *A. terreus* and *A.glaucus* (2 isolates each), Acremonium, *A. nidulans* and Penicillium spp (one isolate each). In tracheal swaps, *A. niger* was the most prevalent isolates (8 isolates) followed by *A. Fumigatus* (6 isolates), *A.flavus* (5 isolates), Zygomycetes (3 isolates) and one isolate of Penicillium. In the examined heart samples, *A. fumigatus* (16 isolates) was the most common isolates followed by *A. flavus* (6 isolates), then *A. niger* (5 isolates), zygomycetes, *A. terreus* and *A. glaucus* (one isolate each). Regarding liver samples, *A. fumigatus* (14 isolates) was the most prevalent, followed by *A. flavus* (10 isolates), *A. niger* (9 isolates), and Zygomycetes (4 isolates). Finally, in kidney samples, *A. niger* was the most prevalent isolates (16) followed by *A. fumigatus* (9 isolates) and *A. flavus* (9 isolates), then Zygomycetes (6 isolates), Acremonium, *A. terreus*, and Alternaria (one isolate each).

Fungal isolates were plated on a blood agar plate for the detection of their hemolytic activity. Fungal isolates displayed various degrees of fungal activities on blood agar plates, 22% of *A. fumigatus* 39% *A. niger* 18.6%, *A. flavus* 33% Zygomycetes displayed hemolysis on blood agar plates. Catalase activity has been revealed by *A. fumigatus* (45.5%), *A. niger* (57%), *A. flavus* (60%), *A. terreus* (20%), Zygomycetes 100%, *Penicillium* 50%. While, amylase activity was displayed by *A. fumigatus* 66%, *A. niger* (64%), *A. flavus* (46.8%), *A. glaucus (33%), Acremonium* (100%), *Penicillium* 50%. In addition, lipase activity was detected by *A. fumigatus* (47%), *A. niger* (66%), *A. flavus* (58%). *A.terreus* (20%), Zygomycetes (11%). Biofilm production in vitro has been displayed by *A. fumigatus* (86.7%), *A. niger* (78.5%), *A. flavus* (79%), *A. terreus* 20%, Acremonium (50%), Penicillium (50%), Zygomycetes (14.8%) (Table 3).

Regarding litter samples, A. *fumigatus*, A. *flavus*, A. *niger,* and *mucor* spp were isolated. Interestingly, *A. niger and A. fumigatus* were common isolates from both birds samples and litter samples. A. *fumigatus* was isolated from straw, deep litter, and rice husk type, while, *A. niger* was isolated from the same samples as well as sawdust. On the other hand, Zygomycetes were isolated from deep litter and sawdust, while *A. flavus* was isolated from rice husk litter. The prevalence of fungi was higher in straw, deep litter, hay than sawdust.

In total, *Aspergillus* species were the most predominate isolates including, *A. fumigatus* (68; 32.69%), *A. niger* (56; 26.92%), *A. flavus* (56; 26.92%), *A. terrus* (5; 2.4%) *A. glaucus*, *A. nidulans* (1; 0.48%), followed by Zygomycetes (27; 12.9%); while the prevalence of *Penicillium, Alternaria*, and *Acremonium* were 0.96%, 0.96%, and 0.48 %, respectively (Table 3).

In total, 49 representative isolates were screened for the presence of three virulence genes. A. *fumigatus* (9 isolates), Zygomycetes (9 isolates), *A. niger* (8 isolates), *A. flavus* (9 isolates), A. *terreus* (5 isolates), A. *glaucus (3 isolates)*, *A. Nidulans* (one isolate), Acremonium (Two isolates), Penicillium (Two isolates), Alternaria (Two isolates). Regarding *fos-1* gene, it was detected in A. *fumigatus* (3 isolates) and *A.flavus* (Two isolates), *rhbA* was detected in *A. fumigatus* (8 isolates) and one isolate of *A. flavus*, while, *Pksp* 2 was determined in *A. niger* (2 isolates) and seven isolates of *A. fumigatus* (Table 4).

The susceptibility testing of antifungal drugs was carried out using the agar dilution method. As it shown in Table 5, Minimum Inhibitory concentration (MIC) for clotrimazole 125 µg /mL with *A. niger* and *A. flavus* and 500 µg /ml on *A. fumigatus* and 250 µg /mL on A. *glaucus* and 1000 µg /mL on Acremonium and Zygomycetes, while, ciclopiroxolamine 125 µg /mL on *A. niger* and *A. terrus* and 1000 µg /ml on *A. flavus*, and 500 µg /mL on *A. fumigatus* and *A. niger*, tioconazole 1000 µg /mL *A. glaucus, A. nidulans and A. flavus* 125 µg /mL on *A. terreus*, fluconazole only affect *Acremonium* with a concentration of 1000µg /Ml. All fungal isolates were resistant to nystatin. *Aspergillus* species and Zygomycetes were resistant to fluconazole. While *Acromonium* isolates

were susceptible to fluconazole (1000µg/mL). Zygomycetes showed more resistance to clotrimazole, tioconazole, ciclopiroxolamine than the relatively more susceptible *Aspergillus* spp. (MIC 125–1000 µg/mL).

Table 1. Oligonucleatide primers sequences used in this study.

Protein	Target gene	Primer sequences	Amplicon
Histidine kinase	*fos-1-F*	ATGCACATATTGCTGGTGGA	424
	fos-1-R	AATCAGGTCTTGTCCCAACG	
Polyketide synthase	*pksP-F*	AGCGACGACTACCGTGAGAT	
	pksP-R	AGAATGGGATCGTTGTCAGC	398
Ras-related protein	*rhbA-F*	TGGGCAAATCGTCTCTTACC	
	rhbA-R	AGTCGAGACGAGCACTAGCC	414

Table 2. Prevalence of different fungal isolates in the tested birds and litter samples.

Samples	*A. Fumigatus*	*A. Niger*	*A. Flavus*	*A. Terrus*	*A. Glaucus*	*A. Nidulance*	*Acremonium*	*Zygomycetes*	*Penicillium*	*Alternaria*	Total	%
Lung	19	15	11	2	2	1	1	6	1	0	58	27.88
Tracheal swap	6	8	5	0	0	0	0	3	1	0	23	11
Liver	14	9	10	1	0	0	0	4	0	0	38	18.26
Kidney	9	16	9	1	0	0	1	6	-	1	43	20.6
Heart	16	5	6	1	1	0	0	5	0	0	34	16.34
Litter	4	3	2	0	0	0	0	3	0	0	12	5.76
Total	68	56	43	5	3	1	2	27	2	1	208	
%	32.7	26.9	20.6	2.4	1.4	0.48	0.96	12.9	0.96	0.48		

Table 3. Screening of fungal isolates for Heamolysin, catalase, lipase, amylase, biofilm production in a total of 208 fungal isolates.

Fungal isolates	Number isolates	Hemolysis No.	Hemolysis %	Catalase No.	Catalase %	Amylase No.	Amylase %	Lipase No.	Lipase %	Biofilm No.	Biofilm %
A.*fumigatus*	68	15	22	31	45.5	45	66	32	47	59	86.7
A.*niger*	56	22	39	32	57	36	64	37	66	44	78,5
A.*flavus*	43	8	18.6	26	60	21	46.8	25	58	34	79
A.*terrus*	5	0	0.0 buy0	1	20	0	0.00	1	20	1	20
A.*nidulance*	1	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
A.*glaucus*	3	0	0.00	0	0.00	1	33	0	0.00	0	0.00
Total aspergillus	177	45	25.42	90	50.85	103	58.19	95	53.67	139	78.53
Acremonium	2	0	0.00	0	0.00	2	100	0	0.00	1	50
Zygomycetes	27	9	33	27	100	9	33	3	11	4	14,8
Penicillium	2	0	0.00	1	50	2	100	0	0.00	1	50
Alternaria	1	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00

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Table 4. The distribution of virulence genes in the tested fungal isolates (49) tested

SPP	Number of isolates	*fos-1*	*pksP*	*rhbA*
A. Fumigatus	9	3	7	8
A. *Flavus*	9	2	-	1
A. *Niger*	8	-	2	-
A. *Terrus*	5	-	-	-
A. *Glaucus*	3	-	-	-
A. *Nidulance*	1	-	-	-
Acremonium	2	-	-	-
Zygomycetes	9	-	-	-
Penicillium	2	-	-	-
Alternaria	1	-	-	-

Table 5. Minimal inhibitory concentration of the tested isolates against antifungal agents.

Drug	*Aspergillus*						*Acremo-nium*	*Zygomyc-etes*
	Fumigatus	*Niger*	*Flavus*	*Terrus*	*Glaucus*	*Nidiulance*		
Clotrimazole	500µg /ml	125µg /ml	125µg /ml	resist	250µg /ml	resist	1000µg /ml	1000µg /ml
Tioconazole	Resist	Resist	1000µg /ml	125µg /ml	1000µg /ml	1000µg /ml	resist	Resist
Ciclopiroxolamine	500µg /ml	500µg /ml	1000µg /ml	125µg /ml	Resist	resist	resist	Resist
Fluconazole	Resist						1000µg /ml	Resist
Nystatine	Resist							

4. DISCUSSION

Mycotic infection in poultry is considered one of the most severe problems affecting poultry resulted in high economic losses due to high morbidity and mortality in young chicken as well as immunosuppression in birds. Mycotic diseases can be expected due to the extensive use of antibiotics preparations in the treatment of many diseases and as feed additives, which enhance mycotic complications.

Litter is one of the primary sources responsible for fungal contamination in poultry farms **[32]**. The process of spreading of litter or after being used and removed from poultry farms represents a serious hazard to the farmworker due to frequent exposure to dust, fungi, and their metabolites during their task **[33, 34]**. Therefore, it is crucial to identify mycoflora of poultry litter to recognize the possibility of occupational exposure of poultry workers to the pathogenic fungal species.

By mycological examination of chicken samples, 196 fungal isolates have been recovered. The prevalence of fungi was higher in lung tissue (27.88%) comparing to the other examined samples, similar to what obtained by **Sajid et al. [35]** and **Abdeltawab et al. [36]**. Aspergillus was the most common genus recovered (84.61%), followed by Zygomycetes (12.9%). In addition to previously mentioned species, other genera were also isolated, namely, Acremonium, Penicillium (0.96 % each), and Alternaria (0.48%) (Table 2). Among Aspergillus genus isolates, *A. fumigatus* was the most frequent (32.96%) followed by *A. niger* (26.92%) then *A. flavus* (20.6%). The reason for the variations in different studies may be due to sample size, environment factors, seasonal variation, nutrition requirements, and virulence factors of these fungi **[37]**.

Aspergillus fumigatus was common isolates from both chicks' organs and litter samples. Aspergillosis has been described either as an acute infection leading to severe outbreaks in young birds or as a chronic condition responsible for low productivity and economic losses in adult birds. Aspergillosis should be suspected when birds suffered from debilitation, respiratory distress, without response to antibiotics treatment . The incidence of *A. fumigatus* infection is usually higher in poultry as the spores of this species are smaller than those of other species of Aspergillus **[38]**. But the role of other Aspergillus species that may affect birds couldn't be ignored **[39]**.

Concerning litter, out of Seven litter samples collected from 7 poultry farms, twelve fungal isolates were recovered, including four fungal species identified as *A. fumigatus* (4 isolates), *A. niger* (3 isolates), *A.flavus (*3 isolates) and three isolates of Zygomycetes. These findings were in line with **Anbu et al. [40],** who stated the most prevalent species

isolated in the litter were *Fusarium solani*, *A. nidulans A. flavus, A. niger,* and other fungal genera. On the other hand, **Viegas et al. [41]** reported *Penicillium* as the most prevalent isolate from poultry litter in addition to *Aspergillus* species. The diversity of litter mycoflora may be contributed to the material choice, litter aging, and handling techniques **[42]**.

The presence of virulence factors confirmed the pathogenicity of the isolates. Pathogenesis of aspergillosis is dependent on various factors of the host (immune status) and virulence factors of the pathogen. The principal mode of action of these virulence factors is as follows cause degradation of tissue carbohydrate (α–amylase) and lipids (lipase), Haemolysin causes lysis of red blood cells **[43]**. Catalase helps in detoxification of reactive oxygen species (ROS) produced by macrophages and neutrophils **[44]**. *Aspergillus* catalase plays a potential role in allowing the organism to escape or to minimize hyphal damage by inactivating hydrogen peroxide **[45]**. Higher prevalence of enzymatic activity was recorded in a study conducted by Zohri et al. [26], where out of eighty isolates of *Aspergillus* species, all of the tested isolates were able to produce catalase and peroxidase enzymes. Meanwhile, 82.5-90% of the fungal isolates could produce protease, lipase, urease, and phospholipase. Whereas 70% of isolates exhibited hemolytic activities. In another study, out of 110 tested isolates 73, 92, and 78 produced protease, lipase, and urease, respectively; meanwhile, 77 of the tested isolates exhibited some hemolytic activities **[46]**.

Interestingly the overall prevalence of the assessed virulence factors, the prevalence was found more in *Aspergillus* species, especially *A.fumigatus* isolated from chicks samples then environmental samples; this could be due to invasiveness nature of *Aspergilli*.

In addition to the evaluated extracellular hydrolytic enzymes in fungal isolate, we used PCR assay for the detection of genes responsible for the virulence of fungi. Three genes were targeted to be tested with PCR. (*fos-1, pksP,* and *rhbA*) which are involved in aspergillus infection such as evasion from the immune response, and the conidial melanin-DHN (pksP/alb1 gene). Our results revealed that *fos-1* was detected in A.*fumigatus* (3) and *A.flavus* (2). While *pksP* detected in A.*fumigatus* (7) and *A.niger* (2). *rhbA* detected in A.*fumigatus* (8) and *A.flavus* (1). The detection of such genes in our isolates confirms the pathogenicity of these species and their important role in pathogenicity.

Many different studies have demonstrated that fungal biofilms show increased levels of resistance against many classes of antifungal drugs, especially azoles and polyenes **[47]**. Studying the ability of biofilm formation is very important as the incidence of mycotic infections has dramatically increased nowadays, which is frequently associated with biofilm formation on implanted biomaterials and/or host surfaces **[48]**. In this study biofilm production as revealed by 86.7% (59/68) *A. fumigatus* , 78.5% (44/56) *A.niger*,79% (34/43) *A.flavus*, 20% (1/5) *A.terreus*, 50%(1/2) Acremonium, 14.8%(4/27) 50% (1/2) Penicillium.

In this study, we investigated the in-vitro efficacy of antifungals against five representative fungal isolates from diseased chicken and litter cases using microdilution assays. As is the case of bacteria, no single antifungal was most effective for all fungi. Clotrimazole has the lowest MIC against *A.niger* and *A. terreus*. Tioconazole has the lowest activity against *A.terreus*. Ciclopiroxolamine also has the lowest MIC against *A.terreus*. Mostly all isolates were resistant to fluconazole and nystatin. Long-term use of azole drugs for aspergillosis is the major reason for the emergence of azole resistance **[49]**. Also, the improper use of fungicides in agriculture results in the increase in azole resistance **[16, 50]**. Interestingly, the high resistant to fluconazole explained also by **Odds et al. [51]** who found that in vitro susceptibility tests in new triazole and fluconazole did not accurately reflect the therapeutic efficacy while the activity of fluconazole in vivo animal model infection was found to be several times greater than the predicted in vitro measures. Besides, earlier studies showed that *A. fumigatus* that resistant to antifungals was observed has the capability of biofilm formation **[52]**.

The MIC determination provides an evidence for nystatin treatment failures in clinical cases of aspergillosis and other isolates. The best effective drug on isolates is clotrimazole followed by ciclopiroxolamine and tioconazole. Following the current study, **Guarro et al. [53]** confirmed that *Acremonium spp.* are generally resistant to the most commonly used antifungal agents.

Conclusion

In conclusion, Effective prevention of mycotic infections throughminimization of stress factors,strict hygienic measures, good litter management practice, and sanitation in brooders and hatcheries should be considered. Control measures like strict biosecurity program, and regular monitoring of fungal infections as well as appropriate use of anti-fungal drugs Should be followed.

Acknowledgment

Conflict of interest statement

No conflict of interest was declared.

Research Ethics Committee Permission

The current research work is permitted to be executed according to standards of Research Ethics committee, Faculty of Veterinary Medicine, Mansoura University.

Authors' contribution

SM collected samples and carried out the experiments; AA wrote the manuscript and shared with SM in conducting experiments, and GY designed the study and reviewed the manuscript. All authors approved the final manuscript for publication.

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