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Research Article

Pathology and Epidemiology of Fungal Infections in Layer Chicken Flocks

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Fungal infections have a key effect on the commercial poultry production and welfare. Infections caused by fungi and their food contaminants are zoonotic and influence food safety. Eggs and cooked meats remain major public health concerns. Therefore, this research is aimed at examining the pathology and understanding the epidemiology of fungal infection in layer chicken flocks. The study was carried out on twenty-layer flocks from Kafrelsheikh Governorate, Egypt, from January 2019 to December 2020. In total, 600 samples were collected from 100 healthy and diseased layer chickens from different organs (skin, liver, lung, kidney, spleen, and ovary). In this work, we present the clinical and pathological characteristics of some fungal pathogens (*Aspergillus* spp. and *Fusarium* spp.) in layer chicken flocks, as they are responsible for reducing the egg production. In total, 19 fungal strains were isolated from individual chickens, and these were analysed to determine the fungal species. The total proportion of fungal infections at the farm level was (3/20) 15%. The main clinical signs were emaciation and mortalities that reached 4.34% ± 0.84. We report the first isolation of *Aspergillus piperis* and *Fusarium* species from the ovary of poultry, which is the main reason for egg retention and multiple numerous nodules of occasional caseating centers in layer ovaries. The histopathological findings of *Aspergillus* infection are indicated by the presence of branched hyphae that tend to be numerous and progressive. Furthermore, we found spherules with multiple endospores of *Fusarium* spp. in the ovaries. Morphological and molecular identification and analysis were performed to confirm the etiological agents.

1. Introduction

Aspergillosis is an important infection that causes severe disease in different animals and humans. It is caused by a class of fungi that are saprophytic and opportunistic in the genus *Aspergillus* [1]. These mycoses were defined several years ago but continue to cause significant economic misfortunes to the poultry commercial sectors, as a result of their direct infection that results in a severe pathological lesion on the host, mycotoxin activity, or the mycotic secondary metabolites which are delivered in grains or poultry feed [2, 3]. Although aspergillosis is associated with diseases of the respiratory system, *Aspergilli* are characterised by the ability to migrate from the lung to other parts of the bird such as the liver and ovary, leading to disease syndromes characterised by acute to chronic infections, which are characterised by suppressed growth and the cessation of egg laying [4].

Aspergillosis has been shown to affect different types of birds, including poultry, ducks, turkeys, game birds, waterfowl, and other birds worldwide. Importantly, in addition to *Aspergillus* species, their spores can survive extreme conditions, and several lines of evidence point to their resistance to disinfectants. *Aspergillus* spp. can enter through eggshells and infect the embryo. These infections are responsible for the death of the embryo or its hatching with a well-

developed pathological lesions. When infected eggs break up, many spores will be released, which will exist in the hatchery system and contaminate all other eggs [5].

The infection by *Aspergillus* in poultry was shown to have an incubation period that ranged from 2–5 days. Aspergillosis in chicken is associated with low morbidity; however, it may reach 12%. In addition, the mortality rate among infected chicks is ranging from 5–50% [6].

Aspergillus fumigatus is the main species associated with Aspergillosis in poultry; nevertheless, other species including *Aspergillus flavus* and *Aspergillus niger*, in addition to *Aspergillus glaucus* and *Aspergillus nidulans*, that can cause single or mixed infections of *Aspergillus* have been implicated in in poultry disease [7–9]. *Aspergillus fumigatus* has small spores compared to other *Aspergillus species,* which may be the reason why *Aspergillus fumigatus* has been implicated in most cases of aspergillosis [10].

Aspergillus spp. is ubiquitous, and aspergillosis may be initiated where environments are favourable for mycological growth. These organisms are normally present in the soil, and organic matter is a major factor that has been implicated in their growth, particularly in warm, damp environments, with a temperature of >25°C including damaged eggs in hatcheries, inadequate ventilation, bad sanitation in the farms [11], and the long-term storing of poultry rations [12, 13]. The aspergillosis diseases more commonly occur in tropical countries [14, 15].

Severe outbreaks associated with hatchery contamination were shown to be responsible for about 15% of chicken mortality through the first two weeks of life and the reduced growth rate in chicks that survived after the infection [16].

Inhalation is considered the leading way for *A. fumigatus* infection in birds [17] and is attributed to the small size of *A. fumigatus* spores. The hallmark of these spores infection is to reach the air sacs and the lungs which are happend following their stucking totally in the nasal cavity and trachea [18]. Importantly, the air sacs serve as the primary infection sites that support the spread of the infection to the lung. Thus, the inhaled air infected with *A. fumigatus* spores reached the subsequent thoracic and abdominal air sacs earlier to bind to the epithelial surfaces in the lungs [19]. In the parenchyma of the lung, the spores become implanted in the atria and some portions of the infundibula in the parabronchi, followed by their uptake by surface phagocytic epithelial cells [20]. Additional research revealed that *A. fumigatus* infection at the air capillaries is initiated by the presence of numerous spores or weakened immune responses. These have been implicated in the development of loosely attached plaques that might become copious as a result of the development of connective tissue. These formed plaques and necrotic debris have been concerned in many of the respiratory and systemic pathological consequences, including obstruction of the trachea or bronchi and/or filling up the air sacs [17]. Infrequently, sporulation happens in the lungs and air sacs [19, 21]. As the hyphae containing fruiting bodies become more compact, it can fill the lumen, which enhances their adhesion to epithelial cells in colonization site followed by penetration of the air sac. This results in the production of serositis and necrosis in the adjacent susceptible organs [22]. In addition to direct extension of the infection through the air sac wall, spreading of mycosis could also arise through a hematogenous way, leading to colonization of hyphae in the tissues and angioinvasion [23]. Host cells also play a role in the spreading mechanism. Additionally, macrophages in the respiratory tract are involved in the process by uptaking the spores which enhances the spread of infection to other organs over the blood and lymphatic streams [24].

Fusarium species (*F.* spp.) are important opportunistic etiological agents of serious infection not only in poultry but also in most birds and animals. *F.* spp. cause illness mainly by producing mycotoxins such as fumonisins, trichothecene, and zearalenone.

Fumonisins (FUM) are a family of mycotoxins discovered in *F. verticillioides* cultures by Gelderblom et al. in 1988 and chemically described by them. Six different FUMs have been found and their structures determined (A1, A2, B1, B2, B3, and B4). Importantly, fumonisin B1 (FB1) was identified as the most frequent type produced by *F. verticillioides*. The FB1 responsible for the hepatic abnormalities in the chicks includes hepatic necrosis which is numerous and multifocal. The FB1 is also responsible for biliary and hepatocellular hyperplasia, as well as enhanced extramedullary hematopoiesis [25].

Trichothecene mycotoxins are a subset of fungal metabolites that have a common basic backbone structure and are secreted by *F. sporotrichioides*. Trichothecenes are the most powerful small-molecule protein synthesis inhibitors, leading to irreversible inhibition of protein synthesis in susceptible cells, followed by a secondary disturbance of DNA and RNA synthesis as the major harmful impact at the cellular level [25].

Zearalenone (ZEN), also known as RAL and F-2 mycotoxin, is a powerful estrogenic metabolite that is formed by *Fusarium* spp., including *F. graminearum*, *F. culmorum*, *F. cerealis*, *F. equiseti*, *F. verticillioides*, and *F. incarnatum.* It was first discovered in *F. graminearum.* This mycotoxin is responsible for the reduction of the egg productivity of chickens by up to 20%, and it also has immunosuppressive effects on their immune systems [25].

This project gives a clear picture of the current state of the fungal infection in the layer chicken industry. We present the clinical and pathological characteristics of some fungal pathogens (*Aspergillus* spp. and *Fusarium* spp.) in layer chicken flocks, as they cause severe mortalities and decreased egg production, which cause huge economic loss. Some of these fungal pathogens are also considered zoonotic diseases and life-threatening to humans. According to our findings, *Aspergillus flavus* was also found in livers and skin lesions. Furthermore, we report the first isolation of *Aspergillus piperis* and *Fusarium* spp. from layer chickens. The isolation was confirmed by histological, morphological, and molecular identification and analysis.

2. Material and Methods

2.1. Study Area and Samples. The work was conducted on 20-layer flocks. Overall, 600 samples were collected from 5

birds of each flock with diseased or healthy chickens ($N = 100$) in Kafrelsheikh governorate, Egypt, from January 2019 to December 2020. After humanely slaughtering the diseased chickens, samples were collected from the internal organs of the layer chickens including the liver, kidney, spleen, ovaries, lung, and affected areas of the skin using a sterile swab and strict aseptic precautions. The collected samples were obtained in two vials each: normal sterile saline and 10% formalin. Saline samples were processed in the mycology lab for the isolation and identification of fungi. The formalin sample was used to prepare histopathology sections. All histological sections from this study were either stained with hematoxylin and eosin (H&E) to show the pathological changes in different organs and periodic acid Schiff (PAS) to demonstrate the fungal hyphae.

2.2. Isolation and Identification of Fungal Species. After homogenizing ten grammes of each internal organ and skin with 90 ml of sterile saline, an aliquot was streaked in triplicate on potato dextrose agar and then incubated for 7–10 days at 30°C. Each sample was streaked on Sabouraud dextrose agar and malt extract agar with chloramphenicol, then incubated at 30° C for 5-7 days to isolate and identify the infected fungi. The identification of fungal species was performed by hyphal morphology imaging under a light microscope and the molecular characterization through the sequencing of 18S rRNA genes.

2.3. Fungal DNA Isolation and Purification. Total DNA was collected from one-week-old fungal plates cultivated on malt extract agar with chloramphenicol using a DNA extraction kit (Qiagen, Hilden, Germany). Mycelial growth from the culture plate was taken and picked using a 2 ml tube containing sterile-distilled water. Two ml of mycelial suspension was transferred to a flask containing one hundred ml of yeast extract with supplements. This fungal mycelial was then incubated for 48 hours with gentle agitation at 28°C and 180 rpm. The collection of the mycelia from the culture was conducted by filtration of the culture using a microcloth under sterile conditions. The mycelia were collected after being washing with sterile distilled water and kept in sterile Petri dishes, then it was freeze-dried overnight at -20°C. Then, the freeze-dried mycelium was minced using a sterile mortar and then collected in a sterile Eppendorf. DNA extraction was conducted using a commercial DNeasy kit as per manufacturer's instruction (Qiagen, Hilden, Germany). In order to confirm the presence of fungal DNA, agarose gel electrophoresis was performed, and the data were analysed using using the transilluminator of a gel documentation system (Bio-Rad, Gel Doc 2000, Budapest, Hungary).

2.4. Polymerase Chain Reaction (PCR). The 18 S ribosomal RNA (rRNA) gene was amplified by PCR according to the primers used by Mirhendi et al. [26]. These primer sets include the forward primer, TCCGTAGGTGAACCTG CGG, and the reverse primer, TCCTCCGCT TAT TGA TAT GC, to amplify a 595–600 bp fragment. PCR was performed using a Taq PCR Master Mix (Qiagen). The reaction was performed in a thermal cycler device (Stratagene, California, United States of America) in the Regional Center for Mycology and Biotechnology at the Al-Azhar University, Cairo, Egypt. The cycling condition included (5 minutes at 94°C) for denaturation, 30 cycles of (20 seconds at 94°C, 30 seconds at an ideal annealing temperature for each amplified piece of DNA, and 60 seconds at 72°C) for annealing/extension reactions, and the final extension stage of one cycle (5 minutes at 72°C) was used. After that, the machine was kept at 4°C.

2.5. Purification of DNA Bands from Gel. DNA bands (including the amplified PCR products and linear plasmids) were cleansed and purified using electrophoresis whereas the samples were run on 1.5% agarose gels.

2.6. DNA Sequencing, Assembling of Nucleotide Sequences, and Phylogenetic Analysis. Three PCR fragments represent the positive samples (3/19) belonged to three different types of *Aspergilli* and were purified from agarose gel electrophoresis and processed for DNA sequencing. The DNA sequences were performed by using the Cy5/Cy5.5 dye primer sequencing kit according to manufacturer's instructions (Visible Genetics Inc., Toronto, ON, Canada) using an open-gene automated DNA sequencing system [27, 28] at the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt. The obtained DNA sequences were exposed to BLAST procedures and genomic databases of the NCBI National Center for Biotechnology NCBI (http://www.ncbi.nlm.nih.gov/html). The alignment yield files were used for drawing phylogenetic neighbor joining and boot strap analysis with 1000 repeat boot strap tests in MEGA X software (http://www.megasoftware.net/) [29].

2.7. Statistical Analysis. The examined 20 flocks were divided into 3 groups: flocks with fungal infection, flocks with infection other than fungal infection, and control normal flocks. The significant difference between the means of mortality rate and egg production among the 3 groups of flocks was identified using ANOVA. Bonferroni post hoc was used to identify the difference between each pair of flocks. The results were considered significant at the alpha level for determination was set at 0.05. Data were analysed using IBM® SPSS® Statistics version 21.0.

3. Results

3.1. Clinical Signs and P.M. Findings. Out of the twenty (58–61-week-old) layer chicken flocks investigated for fungal infection, three flocks developed the following clinical signs and PM lesions that suggest fungal infection in some birds: severe emaciation, reduction in egg production, and mortalities reaching $4.34\% \pm 0.84$. The flocks were divided into three groups, including group one in a normal state, group two with a fungal infection, and group three with other reasons for mortality and a reduction in egg production. Statistical analysis showed a significant difference among the 3 groups of flocks in the mortality proportion at $p < 0.05$ (Figure 1). There are 3 flocks with infectious bronchitis (IB) infections that were removed from the analysis of

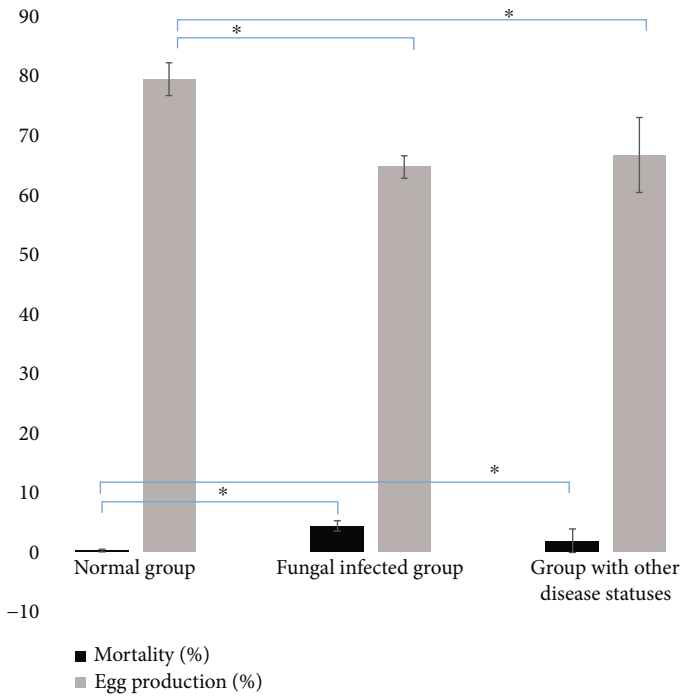
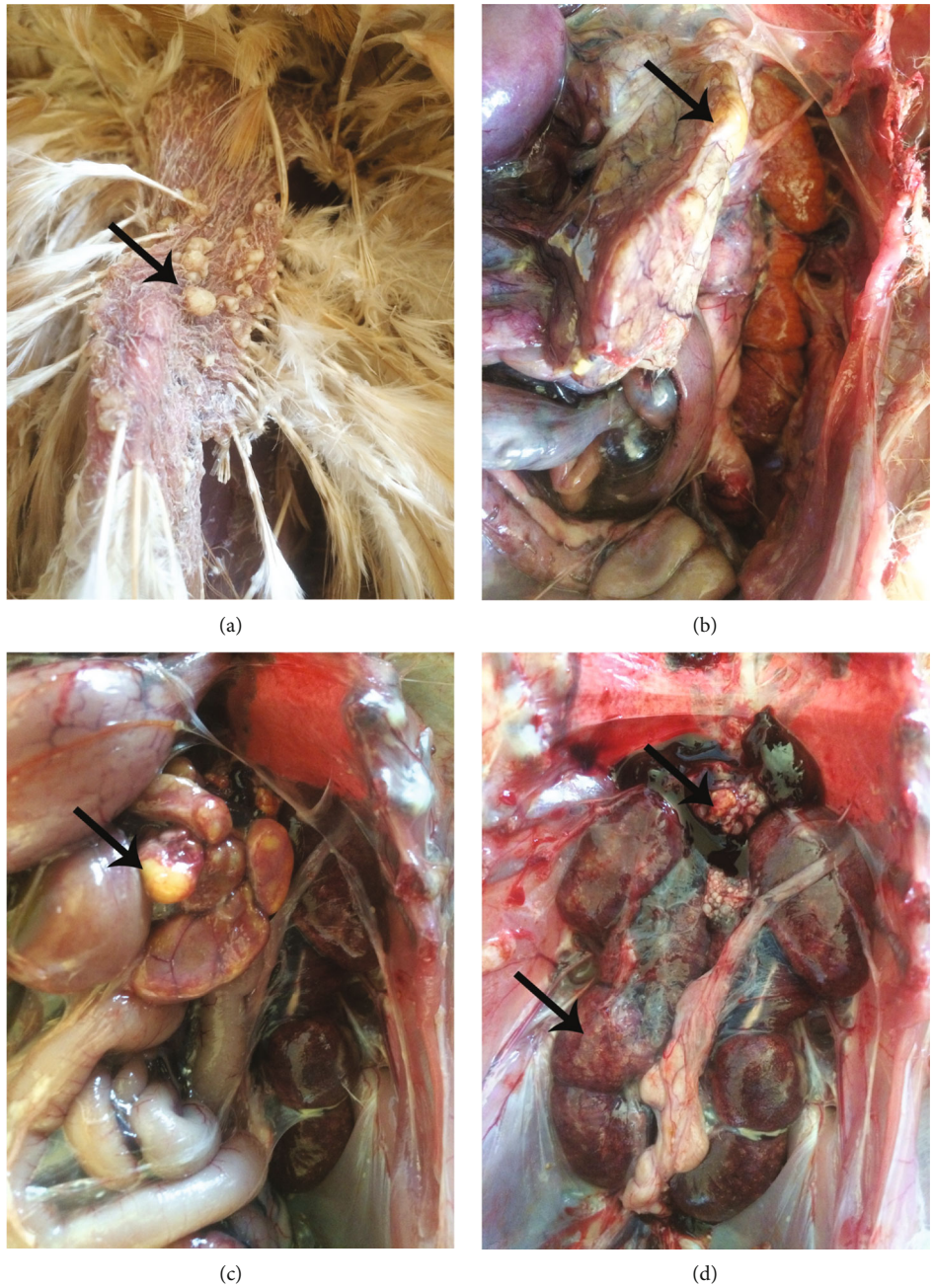


Figure 1: Mortality rate and egg production percentage among normal, fungal infection, and other disease conditions in layer chicken flocks. The mortality rate and egg production in fungal-infected layer flocks were (4.34% ± 0.84 and 64.677% ± 1.86) significantly higher ($^*p < 0.05$) than the mortality rate and egg production in the normal groups (0.2 ± 0.21 and 79.41 ± 2.74). While at the other diseased conditions, there was no significant difference in the rates of mortality and egg production (1.86 ± 2 and 66.69 ± 6.27) compared to the fungal-infected group.

Table 1: Mortalities and egg productions in layer chicken flocks with and without fungal infection in the Kafrelsheikh Province, Northern Egypt.

Flock no.	No. of birds	Age/week	Number of deaths	Mortality (%)	Egg%	Egg number	Aetiology
1	9500	58	320	3.37	66.683	6335	*A. flavus* AMS2 and 3 strains
2	8300	60	404	4.87	63.019	5230	*A. Pipers* AMS1 strain
3	7000	61	334	4.77	64.33	4503	*Fusarium* spp.
4	1000	61	6	0.6	75.3	753	Nutritional
5	2000	61	4	0.2	72.65	1453	Nutritional
6	6000	61	80	1.33	53.33	3200	Infectious coryza
7	3500	60	48	1.37	64.29	2250	Infectious coryza
8	2000	55	45	2.25	57.5	1150	Egg drop syndrome
9	6000	60	22	0.37	72.5	4350	Nutritional
10	12000	61	820	6.83	63.58	7630	ILT
11	25000	61	12	0.05	80.92	20230	Normal
12	30000	62	27	0.09	82.33	24700	Normal
13	5000	59	160	3.2	59.04	2952	Colibacillosis
14	3000	57	923	30.77	51.13	1534	IB
15	15000	61	4500	30	63.33	9500	Viral (IB)
16	10000	61	15	0.15	76.2	7620	Normal
17	10000	61	30	0.3	70	7000	Nutritional
18	20000	62	420	2.1	65.33	13065	Bacterial (colibacillosis)
19	12000	59	4020	33.5	62.08	7450	Viral (IB)
20	10000	59	50	0.5	78.2	7820	Normal



(a) (b) (c) (d)

Figure 2: P.M. lesions of fungal infection in sixty two-week-old layer chickens. (a) Multiple grayish-yellow plaques on the skin (arrow). (b) Liver showing diffuse grayish-yellow necrotic areas (arrow). (c) Liver showing the locally extensive necrotic area (arrow). (d) Kidney showing discrete, minute grayish necrotic areas (arrow).

mortalities due to very high mortality proportions among these 3 flocks. The mortality proportion was significantly higher in flocks with fungal infection than in normal flocks at $p < 0.05$. Also, the proportion of mortality in flocks with fungal infection was higher than in flocks with other infections, but this was not a significant finding. Importantly, there was a significant difference in the mean of egg production between the 3 types of flocks at $p < 0.05$. The egg production among flocks with fungal infection and flocks with other disease-causing agents was significantly reduced compared to control normal flocks at $p < 0.05$. Moreover, there was no significant difference in egg production between flocks with disease status, either flocks with fungal infection or those with other disease statuses (Figure 1 and Table 1).

The main growth lesions observed in the sacrificed 61-week-old chickens that were positive for fungal infection were cutaneous grayish-white nodules on the brisket, subcutaneous hemorrhage, and severe liver enlargement, which was congested and covered with a serosanguinous membrane in some cases, without any masses, and sections were very friable. The spleen was enlarged with a soft section, while the kidney showed severe enlargement

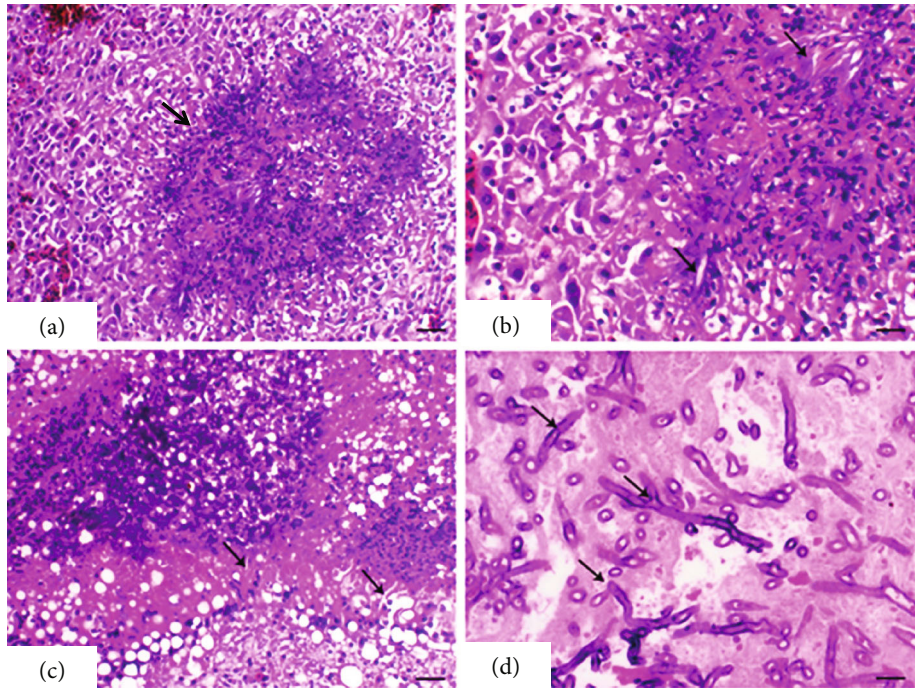


Figure 3: Hepatic and ovarian sections were stained with H&E and PAS stains. (a, b) H&E stain of liver tissue, showing a focal granulomatous lesion with a central necrotic area and definite regular spaces (arrows) surrounding hepatocytes that exhibit necrobiotic changes. (c) H&E stain of the ovary showing the locally extensive granulomatous area surrounding eosinophilic amorphous necrotic tissues (arrows). (d) PAS stain showing septated, branched hyphae suggestive of *Aspergilli* (arrows).

Table 2: The total fungal species isolated from tissue organs collected from 20-layer chicken flocks in Kafr El-Sheikh Province, Northern Egypt.

Fungal strains	Number of isolates	Number of chickens	Organs of isolation
A. Pipers AMS1	4	5	Ovary
A. flavus AMS2	5	5	Skin, liver, kidney, spleen
A. flavus AMS3	5		Liver, kidney, spleen
Fusarium spp.	5	5	Ovary
Negative samples	0	85	Skin, liver, lung kidney, ovary, and spleen
Total	19	100	600

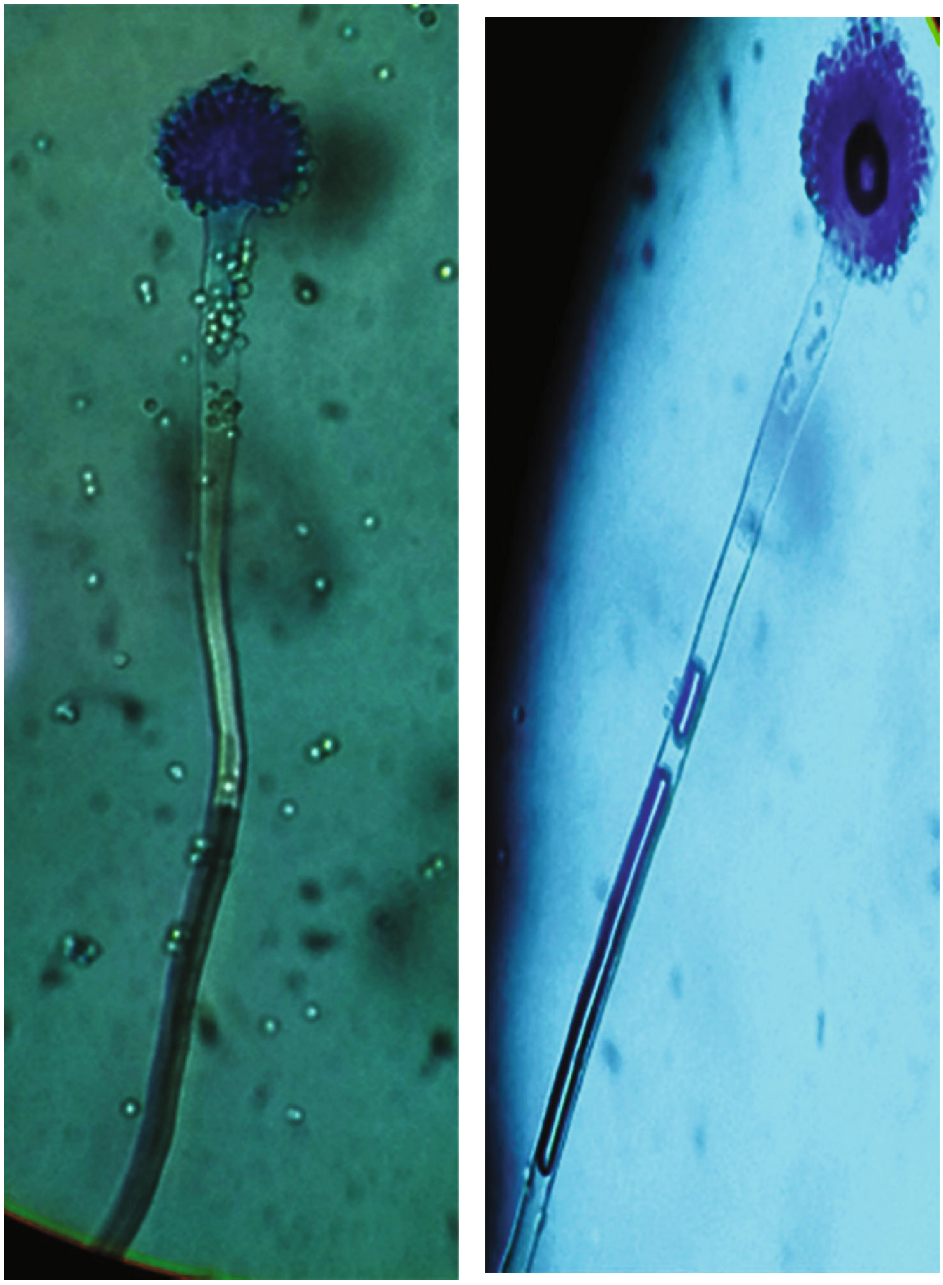
with a remarkable bully kidney and gouty picture in the cut section (Figure 2).

Ovarian lesions include egg retention, numerous nodules, and the occurrence of caseating centers. The lung was congested with petechial hemorrhage. The bursa and sciatic nerve were of normal size; examples of which are shown in Figure 2. These signs and lesions were associated with a high mortality average of 4.34 ± 0.84.

3.2. Histopathological Findings. Most liver samples have shown multifocal pyogranulomas associated with many elements of the central hyphal elements, peripheral macrophages, and giant cells. Marked vasculitis accompanied by the infiltration of inflammatory cells of perivascular mononuclear and plasma cells. Furthermore, pyogranulomas were seen in different organs, including the ovaries, lungs, and skin. The PAS staining of the presented samples indicates branched and separated hyphae (Figure 3).

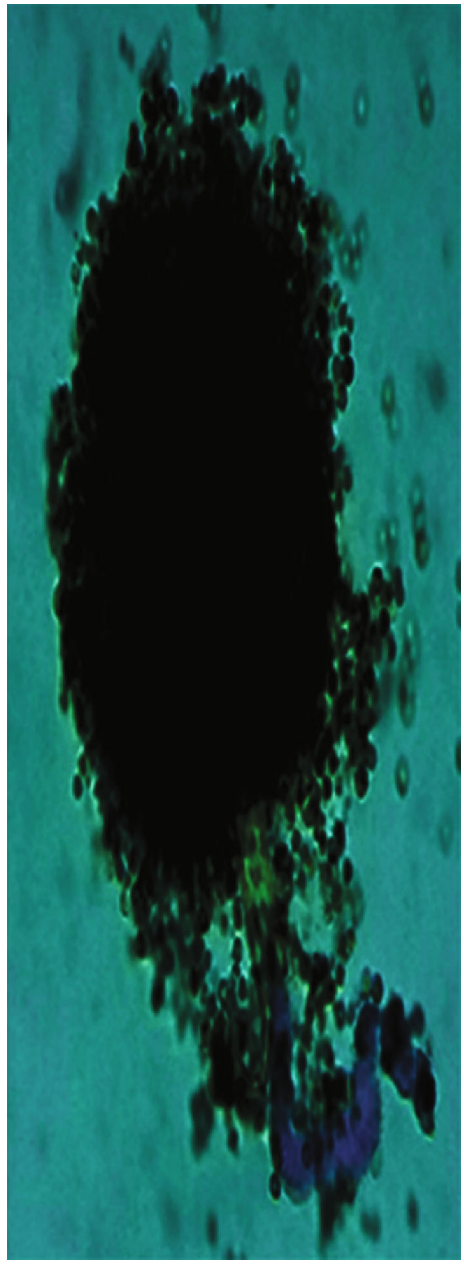
In the presence of *Fusarium* spp. and *Aspergillus* spp., the ovaries showed pyogranulomatous lesions mixed with fungal hyphae (Figure 3). Kidneys had hemorrhage, gout, and interstitial nephritis (data not shown). The spleen showed severe necrosis associated with marked lymphoid depletion (data not shown).

3.3. Mycological, Molecular Characterization of Isolated Fungi. We examined 20-layer chicken flocks for fungal infection by culturing 600 specimens from the liver, skin, ovary, kidney, lung, and spleen of five randomly selected birds per flock for fungal isolation and genotyping. Mycological and molecular investigations of these samples indicated that five samples from the skin were positive for *A. flavus* AMS2 (Table 2 and Figure 3). *A. flavus* AMS2 and *A. flavus* AMS3 were isolated from liver, kidney, and spleen, whereas *A. pipers* AMS1 was collected from four diseased ovaries of fungal-positive layer chickens (Table 2 and Figure 4). The



(a) (b)

Figure 4: Continued.

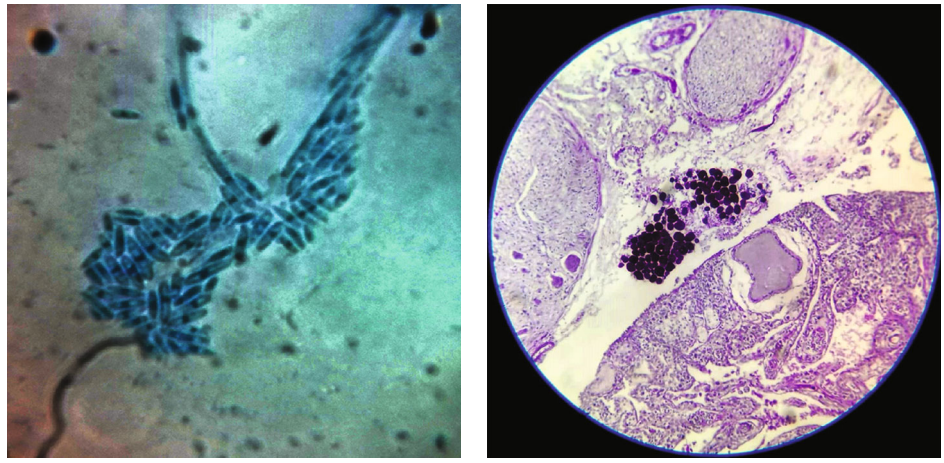


(c)

Figure 4: Microscopical identification of *Aspergilli* spp. from layer chicken flocks. Microscopy of *Aspergilli* spp. with lactophenol cotton blue. (a) *Aspergillus flavus* isolated from layers liver. (b) Aspergillus *flavus* isolated from the layers skin. (c) *Aspergillus pipers* isolated from the layers ovary.

Fusarium spp. was only isolated from the ovaries of five chicken of one-layer flock (Table 2 and Figure 5). To confirm the presence or absence of bacterial infection, the samples from the skin and internal organ granulomas were taken and streaked on MacConkey agar under aerobic and an anaerobic conditions. Our results indicated there were no bacterial organisms.

The molecular identification of isolated *Aspergilli* was carried out according to the previously established criteria defined by Mirhendi et al. [26]. We carried out PCR for the targeted genes (18S rRNA) from DNA preparations. The right size of the amplified DNA target gene was extracted from the agarose gel, and on this basis, we found an amplicon bands of expected size of approximately 595–600 base pairs (bp). The gene sequences and phylogenetic analysis were investigated for the relationship with reference database sequences from GenBank. To test this, the pairwise comparison was used to determine if there were differences. Our results demonstrated that there was a low distance among the sequences from the targeted three fungal species of *Aspergillus* related with reference sequences from GenBank which is indicated in Figures 6 and 7 between our



(a) (b)

Figure 5: Identification of *Fusarium* spp. isolated from layer chicken flocks. (a) *Fusarium* spp. hyphae isolated from ovary and stained with lactophenol cotton blue. (b) Spherules with multiple endospores inside. PAS.

Aspergillus flavus isolate AMS3 that was isolated from layer chickens liver with GenBank accession number MW522551.1, was 100% with *_Aspergillus_flavus_*isolate_SS2021_ (GenBank accession no. MW485934.1), *Aspergillus_flav*us_isolate_53_2H2 (GenBank accession no. KY859367.1), *Aspergillus_flavus_*strain_GFR40 (GenBank accession no. MT447545.1), *Aspergillus_flavus_*strain_IM21 (accession no. KX011593.1), *Aspergillus_flavus_*isolate H8 (accession no. LC513851.1), and *Aspergillus_flavus_*isolate_isolate_PB322 (accession number MN006669.1).

The sequence of our *Aspergillus flavus* isolates AMS 2 that isolated from layer chickens skin with gene bank accession number MW522551.1 was examined, as shown in Figures 6 and 7, the ratio of relation between the *Aspergillus_flav*us AMS 2 sequence obtained in this study has been mapped to 99% identical with *Aspergillus_flavus* GenBank accession no. MK091395.1, *_Aspergillus_flavus* GenBank accession no. JX157882.1, *Aspergillus_flav*us_isolate_53_2H2 (GenBank accession no. KY859367.1), and *Aspergillus_flavus_*strain_GFR40 (GenBank accession no. MT447545.1), while it showed a 98% identity with *_Aspergillus_flavus_*isolate H8 (accession no. LC513851.1).

The *Aspergillus piperis* isolate AMS1, isolated from the chicken ovary in this study with the gene bank accession number MW165829.1, showed 99% identity with previous sequences in the GenBank Aspergillus piperis clone SF 1001 (accession number MT530277.1), the *Aspergillus pip*eris clone EF570 (accession number MT529219.1), the Aspergillus niger isolate 34 5H2 (accession number KY859364.1) and the *Aspergillus tubingensis* strain mktt13 (accession number MT318170.1) (Figures 6 and 7).

4. Discussion

Poultry generates the income needed for producers and the economy while providing safe products for our tables. Fungal infections represent a common mismanagement problem and cause high mortalities in chickens. A previous study has demonstrated that environmental factors play a critical role during the development of the infection, including the number of spores to which the bird is exposed, poor sanitation in the house, and immunosuppression, as well as food contaminated with faces where these conditions would be advantageous for fungal infections [30].

In this study, we found that 15% of the layer flocks had fungal infections that cause emaciation and mortalities that reach up to $4.34 \pm 0.84\%$. Remarkably, these prevalences and mortalities are higher than those reported in several previous studies [31]. In previous studies, Kitsopanidis and Manos [32] have demonstrated that the mortality rate is more than 2 to 5% in cases of fungal infection in layer chickens, whereas North and Bell [33] have shown that the mortality rate of the egg layer type is more than 10%. Mortality at any stage of life impelled us to speculate on the direct role of fungal infections in the layer chicken industry. Our findings suggest that farmers and veterinarians in the study area did not use standard sanitation and management protocols for the prevention of fungal infections. Moreover, we report *A. flavus*, *A. pipers*, and *Fusarium* spp. were the predominant etiological agents associated with the flocks under investigation as a result of the low management regimes demonstrated by several earlier studies [34]. Furthermore, we have shown that fungal pathogens show obvious clinical manifestations, including depression, severe emaciation, ruffled feathers, a marked decrease in egg production, and mortalities that reach to 4.34 ± 0.84.

As previous studies have demonstrated, the infection caused by *A. fumigatus* causes severe respiratory disease in commercial poultry, while *A. flavus* typically does not cause any respiratory signs [34, 35]. Following our observations of *A. flavus,* which infects layered chickens and has shown no respiratory signs or lesions, we hypothesized that the infection occurred through contaminated food but not through the respiratory passage, and then fungal organisms are expected to spread in the different organs of the infected bird. These results also are in agreement with Barton et al., Barton et al., and Kunkle et al. [7, 30, 36].

Figure 6: Phylogenetic neighbor-joining tree of the 18S rRNA gene sequences with 1000 repeats bootstrap. The tree was separated into 2 groups: group 1, in which the isolates *Aspergillus piperis* isolate AMS1 identified in this study (blue circle) was aligned with SF 1001and DF09002 obtained from GenBank. Group 2, in which the *Aspergillus flavus* isolate AMS3 and *Aspergillus flavus* isolates AMS2 were identified in this study and (red circle) are aligned with ZMGL1 and GFR40 retrieved from GenBank.

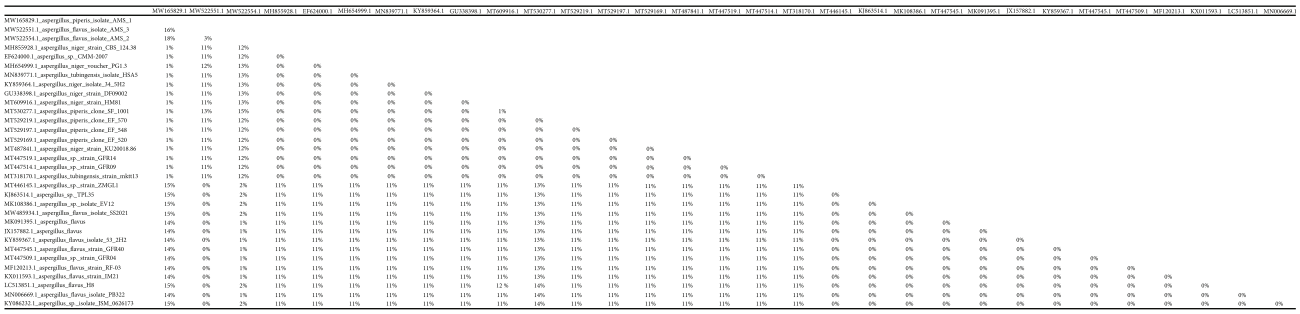


Figure 7: Diversity percent between *Aspergilli* spp. identified in this study and other strains from GenBank. Diversity was calculated by the MEGA X software.

Previous studies have shown that *A. flavus* was isolated from natural infections including the skin [37]. The data obtained from our study showed grayish-white skin nodules on the brisket and subcutaneous haemorrhage associated with dumpiness and immune suppression.

Indeed, numerous *A. flavus* were associated with the infection of the ovaries, kidneys, liver, and spleen of the infected chickens, suggesting the spread of fungi inside the body by systemic way of infection. A weakened immune system is also considered as a significant risk factor in initiating aspergillosis in both people and animals [36, 38].

Previously, Martin et al. [34] have shown that the affected pullets infected with *A. flavus* were dehydrated, emaciated, and had granulomas of different sizes distributed in the liver. These granulomas were single or multiple and contained intralesional septate and branched mycological hyphae. Pathological damages were widespread in the kidneys. Our findings were mostly compatible with those found in the studies shown by Martin et al. [34]. Unlike Martin et al. [34] who reported that *A. flavus* infection occurred in broiler chickens, the bursa and sciatic nerve were not affected in our study. These results suggest that the bursa does not play a role in immunity at this age in layer chickens.

In this study, we used primers against the 18S ribosomal RNA (rRNA) gene as a marker for the diagnosis of *Aspergillus* spp. Our objective was to investigate whether it was a single or mixed infection by sequencing of *Aspergilli*. We found three different species, including *A. flavus*, *A. pipers*, and *Fusarium* spp. Several previous studies concluded that mixed *Aspergilli* infections in equine eyes [39]. We found two strains of *A. flavus*: one from the skin and one of the internal organs from the same flock. The sequence analysis of the two different strains of *A. flavus* indicated a 3% difference in the sequenced 18S rRNA genes, indicating that the origin of these two strains was almost the same with 3 mutations. This suggests that the presence of a single origin of infection is in addition to the more spread of the strains that could be linked to the marketing of live birds, and as a consequence, this increases the reassortment activities of *A. flavus* to adapt to infection in different organs.

A strain of *A. pipers* was isolated only from the ovaries of the second flock, and *Fusarium* spp. was isolated from the ovaries of the third flock.

Aspergillosis is of public health importance because it causes severe human disease as a result of inhalation or ingestion of spores and mycotoxins produced by *Aspergillus* spp. from infected rations and litter, insufficient sanitation, unsuitable hygienic conditions, and by eating undercooked contaminated poultry [40].

5. Conclusion

This study used a focused approach to give a clear idea of the present situation of the fungal infection in the layer chicken business. Therefore, it is prudent that the capacity for fungal disease diagnosis, treatment, and prevention protocols should be developed and shared among farmers for minor economic burdens and human health risks. Limiting this disease burden should not only progress the welfare of managed layers but also provide additional approaches to meet the WHO Millennium Progress Goal of eliminating extreme poverty and starvation. In addition, we report the first isolation of *Aspergillus piperis* and *Fusarium* spp. from the ovary of poultry, which are thought to be the main etiological agents responsible for egg retention, as well as multiple numerous nodules from rare caseating centers.

Data Availability

All data generated during this study are included in this published article. Sequence data sets generated during the current study are available in the DDBJ/ENA/Gen Bank repository under accession numbers. (1) MW165829 (https://www.ncbi.nlm.nih.gov/nuccore/MW165829/) (2) MW522551 (https://www.ncbi.nlm.nih.gov/nuccore/MW522551) (3) MW522554 (https://www.ncbi.nlm.nih.gov/nuccore/MW522554).

Ethical Approval

The ethical approval has been obtained from the supervision of Research, Publication, and Ethics of the Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt, with code number KFS-2019/1, which complies with all relevant Egyptian legislation in research and publications.

Conflicts of Interest

The authors declare that they did not have any conflict of interests.

Authors' Contributions

H.E., A.A., and A.T. were involved in the origin of the research idea and the plan of the methodology, in supervision, in performing data analysis and interpretation, and in drafting and preparing the manuscript for publication and revision. All authors have underwritten the investigational study and design, and all authors have consented to the publication. All authors have read and approved the final manuscript.

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