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Genotypic Characterization of Fungal Species Isolated From Broiler Breeder Chickens, Dead-In-Shell and Hatched Chicks

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Abstract
To investigate the prevalence of fungal agents in local Egyptian broiler breeder chicken's premises, tracheal and cloacal swabs from chickens, feed, and water samples were collected. The targeted breed's dead-in-shell eggs and newly hatched chick's samples were also tested. All fungal isolates were morphologically typed and the predominant fungal species were further subjected to molecular typing using PCR-RFLP and gene sequence analysis of the β-tubulin gene. Results revealed a high prevalence of fungal isolates in tracheal and cloacal swabs (39.3 - 48.1%) and feed and water samples (37.5% and 28.6%, respectively). Fungal isolation rates in dead-in-shell eggs of all breeds were high except in Dahaby breed. Aspergillus species including *A. niger*, *A. flavus*, and *A. terreus* were the predominantly isolated fungi from all collected samples. The ß-Tubulin genes PCR-RFLP of selected Aspergillus isolates showed a characteristic restriction pattern for each species; however, the method was unable to distinguish between strains. The β-tubulin gene phylogenetic and sequence analysis of selected *A. flavus*, and *A. terreus* from breeder chickens and their hatching chicks indicated their relatedness to isolates from bronchopulmonary Aspergillosis in humans in the Middle East. In conclusion, the Aspergillus species remains the most prevalent fungi in breeder chickens, their incubated eggs and hatched chicks indicating their widespread in hatcheries. The PCR-RFLP is an easy tool to discriminate between Aspergilli species, however, the β-tubulin sequence analysis more descriptive of potential sources of fungal contamination. Further epidemiological studies are needed to monitor avian and human Aspergilli in poultry houses with a special focus on antifungal drug-resistant strains.

Introduction
Poultry production represents an important source for animal protein in Egypt. Therefore, efforts focused on minimizing the economic losses caused by various infectious agents. In poultry, the fungal infections were described mainly as a respiratory affection. Aspergillosis generally occurs in young birds resulting in high morbidity and mortality (Beernaert *et al.*, 2010). The chronic form is sporadic causing lesser mortality and generally affect older birds especially breeder chickens especially under

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poor husbandry conditions (Arné *et al.*, 2011). Infection by *Aspergillus* species has been reported in almost all domestic birds including broiler breeders (Martin *et al.*, 2007), grower chickens (Akan *et al.*, 2002), and turkey poults (Stoute *et al.*, 2009).

Previous studies showed that fungal isolates of the *Aspergillus* spp. remain the most prevalent fungal species isolated from local Egyptian breeder chicken's dead-in-shell eggs (66.9%), in which *A. fumigatus*, and *A. niger* were the major fungi being isolated (Ahmed, 2015). Dead-in-shell chicken embryos constitute one of several factors accounting for lowered hatchability of incubated eggs. Hatchery losses associated with embryonic mortality may result from non-specific bacterial infections of incubated eggs (Abd El-Galil *et al.*, 1995; Moustafa, 1995; Smith and Rehberger, 2018).

The eggs would be susceptible to infection by numerous infectious organisms. Even though the eggshell appears solid, it contains microscopic pores that can allow liquids and organisms of small enough size to enter the egg. Moreover, embryonated eggs were used as an infection model for virulent *A. fumigatus* isolates instead of the mouse model. The fungus caused embryonic death by invasion of embryonic membranes and blood vessels (Jacobsen *et al.*, 2010; Jacobsen *et al.*, 2012). This indicates the ability of these fungi to invade the eggs whenever it is possible with the potential of causing hatchability problems.

Despite frequent evidence of the ability of the fungi and/or their byproducts to invade the embryonated eggs and causing embryo mortality, very limited literature is available on epidemiology in poultry houses in Egypt and potential sources of contamination. Recent molecular techniques have been developed to overcome the limitations of classical morphological and microscopical procedures. These techniques are beneficial for understanding the fungal pathogens epidemiology in animals and human beings (Wang, 2012).

The current study designed to investigate the prevalence of fungal species in local Egyptian breeder chickens, their dead-in-shell eggs, and hatched chicks. Genetic characterization using PCR-RFLP and gene sequencing and sequence analysis of the β-tubulin gene were employed for genetic identification of fungal isolates.

Materials and Methods

Sampling and samples processing

All experiments were conducted according to Animal Research Ethics Guidelines at the faculty of veterinary medicine, Beni-Suef University, Egypt. One-hundred and seventy-four samples were collected from different broiler breeder chickens (26 - 40-week-old), feed, water, incubators, dead-in-shell embryos, and hatched chicks of different local Egyptian chicken breeds in the period between March 2016 to February 2017. The samples were obtained from an automatic hatchery (El-Azab Integrative Poultry Project, Fayoum Governorate, Egypt). The dead-in-shell eggs samples were a pooled sample from yolk sac, lung, and liver. Similarly, hatched chicks were necropsied just after hatching and lung as well as liver tissues were collected. All samples were transferred directly to the laboratory of Bacteriology, Mycology and Immunology Department, Faculty of Veterinary Medicine, Beni-Suef University for mycological examinations.

For dead-in-shell egg samples, the eggshell was disinfected with 70% ethyl alcohol. A sufficient area of the shell was removed around the air sac with a pair of sterile scissors. The egg contents were transferred into sterile Petri dishes. Loop full of the yolk sac, lung as well as liver were used for fungal isolation. Hatched chicks were euthanized to collect lung and liver samples aseptically.

Morphotypic characterization of fungal agents

Samples were inoculated into Sabouraud's dextrose broth and incubated for 3-5 days at 27°C. A loop full of selective broth was plated onto Sabouraud's dextrose agar that previously prepared with adding chloramphenicol 50 mg/liter dissolved in 10 mL ethyl alcohol. Incubate for 3-5 days at 27°C. Colony morphology of fungal isolates was conducted according to Rippon (1988). For microscopical examination, fungal isolates were stained with lactophenol cotton blue stain (Cruickshank *et al.*, 1975).

Genotypic characterization of fungal isolates

PCR-RFLP of ß-tubulin (*Ben*A) gene of fungal isolates

For DNA extraction, about 100 mg of fungal samples were placed into liquid nitrogen and ground thoroughly with a mortar and pestle. The DNA was extracted using Gene-JET

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Genomic DNA Purification Mini Kit (Thermo Scientific, Lithuania) according to the manufacturer instructions. Oligonucleotide primers targeting the ß-tubulin (*Ben*A) gene (forward: 5'-AATTggTGCCgCTTTCTgg-3' and reverse: 5'-AgTTgTCgggACggAATAg-3') were used as previously described (Staab *et al.*, 2009).

The reaction was performed in a volume of 50 µL consisting of 25 µL of 2X *DreamTaq* PCR Master Mix, 1µL of 20 pMol forward and reverse primers, 5µL of DNA extract and the volume was completed to 50µL using sterile deionized water. The thermal profile consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec., annealing at 55°C for 30 sec., and extension at 72°C for 45 sec. final extension was performed at 72°C for 10 min. Amplified products in both reactions were visualized by 1.5% agarose gel electrophoresis in TBE buffer, stained with 0.5mg/mL ethidium bromide solution. The amplified ß-tubulin (*Ben*A) gene PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen, Germany) according to the manufacturer instructions.

The RFLP of the β-tubulin region PCR products of different fungal isolates was performed (Staab *et al.*, 2009). Briefly, the reaction was performed using 10 µL of the PCR purified products in a 50µL reaction volume containing 1× NE Buffer 1 (pH 7.0), 100 µg/mL bovine serum albumin, and 1.0 U of *BccI* enzyme (New England Biolabs) in a water bath at 37°C for 2 hrs. The resulting DNA fragments were separated on a 3.5% agarose gel and visualized by staining with ethidium bromide.

β-tubulin genes sequencing and sequence analysis

For gene sequencing, the purified PCR products of selected fungal isolates from breeder chickens and their corresponding hatching chicks (Table 1) were sequenced in forward and reverse directions at the Animal Health Research Institute (AHRI), Egypt. A BLAST® analysis was initially performed to establish sequence identity to GenBank accessions (Altschul *et al.*, 2009). Pairwise sequence comparisons were performed using the Mega 6 alignment program (Tamura *et al.*, 2013). Phylogenetic relationships through a bootstrap trial of 1000 were determined with the MEGA version 6 using the Clustal W alignment algorithm and neighbor-joining method for tree construction.

Table 1. Fungal isolates selected for β-tubulin gene sequencing

Fungus	Source	
	Breeder chickens	Hatched chicks
A. flavus	- Dokki tracheal swab (*A. flavus* EGY-LCK-001) - Sainaa tracheal swab (*A. flavus* EGY-LCK-002)	- Dokki liver sample (*A. flavus* EGY-HCK-003) - Sainaa lung sample (*A. flavus* EGY-HCK-004)
A. niger	- Sainaa cloacal swab (*A. niger* EGY-LCK-001)	- Sainaa liver sample (*A. niger* EGY-HCK-002)
A. terreus	- Dokki tracheal swab (*A. terreus* EGY-LCK-001)	- Dokki lung sample (*A. terreus* EGY-HCK-002

Table 2. Prevalence fungi in breeder chickens, feed, water, incubated eggs and hatched chicks

Chicken breed	Breeder chicken farms				Incubators & Eggs		Hatched chicks	
	Tracheal swab	Cloacal swab	Feed	Water	Incubator	Dead eggs	Lung	Liver
Dokki	3/7 [a] (42.8%)	2/7 (28.6%)	0/2	0/2	1/2 (50%)	12/15 (80%)	5/5 (100%)	5/5 (100%)
Sainaa	0/7	2/7 (28.6%)	1/2 (50%)	1/1 (100%)	1/2 (50%)	12/15 (80%)	5/5 (100%)	4/5 (80%)
Gemeza	4/7 (57.1%)	3/6 (50.0%)	1/2 (50%)	1/2 (50%)	NT[b]	3/15 (20%)	2/5 (40%)	0/5 (0%)
Dahaby	4/7 (14.2%)	6/7 (85.7%)	1/2 (50%)	0/2	NT	0/15	2/5 (40%)	1/5 (20%)
Total	11/28 (39.3%)	13/27 (48.1%)	3/8 (37.5%)	2/7 (28.6%)	2/4 (50.0%)	27/60 (45.0%)	14/20 (70.0%)	10/20 (50.0%)

[a] positive/total tested
[b] NT; not tested

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Results

Prevalence of fungi

Based on morphtypying, isolation rates from tracheal and cloacal swabs collected from broiler breeder chickens are high (39.3-48.1%) except for the Sainaa breed that showed lower isolation rated especially from tracheal swabs (Table 2, Figure 1). In dead-in-shell and hatched chick's samples from the 4 breeds, higher isolation rates (80-100%) of fungi were observed in both from dead-in-shell eggs and hatched chicks of Dokki and Sainaa breeds. In contrast, the Dahaby and Gemeza breed dead-in-shell eggs and hatched chick's organ samples showed lower rates (0-20% and 0-40%, respectively).

The most prevalent fungi in tracheal swabs were *A. niger* and *Zygomycetes*, while *A. terreus* and *A. flavus* showed a similar rate of 4.8%. *A. terreus* and *Zygomycetes* were the predominantly isolated fungi from cloacal swabs followed by both *A. flavus* and *A. niger*. *Cladosporium* spp. was isolated only from cloacal swabs with a very low rate (2.5%). *A. terreus* was the predominant strain in feed samples tested in contrast to higher isolation rates of *A. niger* and *Zygomycetes* from water samples. *A. flavus* was not isolated from either feed or water samples (Table 3). In incubators, only Zygomycetes species was isolated, however, in dead-in-shell eggs samples, *A. flavus* was the most isolated species with a rate of 20%, followed by *A. niger* and *A. terreus*. In both hatched chick's lung and liver organ samples the highest isolation rates were for *A. niger* followed by *A. flavus* then *A. terreus*.

Table 3. Fungal species isolated from breeder chickens, feed, water, incubated eggs and hatched chicks

Fungal species	Breeder chickens				Incubators		Hatched chicks		Total (No. 174)
	Tracheal swab (No.28)	Cloacal swab (No.27)	Feed (No.8)	Water (No.7)	Incubator (No.4)	Dead-in-shell (No.60)	Lung (No.20)	Liver (No.20)	
A. terreus	2 (4.8%)	7 (17.5%)	3 (27.3%)	0	0	10 (16.7%)	3 (15%)	1 (5.0%)	26 (14.9%)
A. flavus	2 (4.8%)	4 (10.0%)	0	0	0	12 (20.0%)	4 (20%)	5 (25.0%)	27 (15.5%)
A. niger	6 (14.3%)	5 (12.5%)	1 (9.1%)	2 (18.2%)	0	4 (6.7%)	9 (45%)	7 (35.0%)	32 (18.4%)
Zygomycetes	5 (11.9%)	5 (12.5%)	1 (9.1%)	1 (9.1%)	2 (50.0%)	1 (1.7%)	0	0	15 (8.6 %)
Cladosporium sp.	0	1 (2.5%)	0	0	0	1 (1.7%)	1 (2.5%)	0	3 (1.7%)

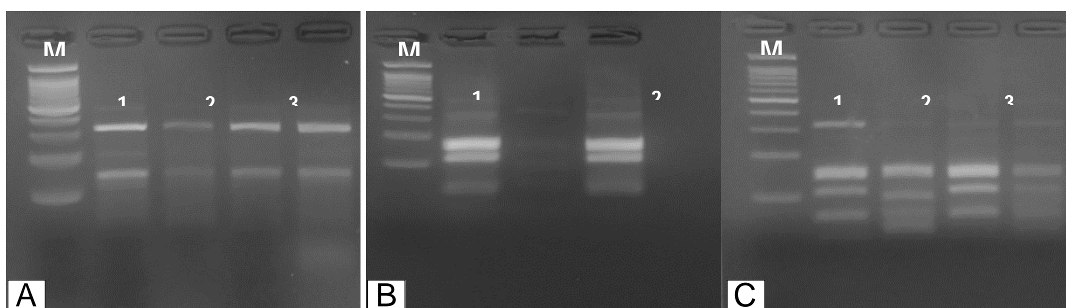

Figure 1. Gel electrophoresis of *BccI* restriction enzyme digested ß-tubulin gene amplicons. M; 100 bp DNA ladder (A) A. *flavus* "lanes 1 and 2 breeder isolates, lanes 3 and 4 hatched chicks isolates". (B) A. *niger* "lane 1 breeder isolate, lane 2 hatched chicks isolates". (C) A. *terreus* "lanes 1 and 2 breeder isolates, lanes 3 and 4 hatched chicks isolates".

PCR-RFLP of ß-tubulin (*benA*) gene of isolated Aspergilli

The ß-tubulin gene fragment (492bp) was successfully amplified for of 7 samples from *A. flavus* isolates, 4 out of 7 samples from *A. terreus*, and 4 out of 4 selected *A. niger* isolates. Selected isolates represented both breeder chicken and their hatched chick's isolates (Data not shown). The *BccI* restriction enzyme analyses of the Aspergillus species. The *A. flavus* restriction yielded characteristic fragments of 349 and 107 bp (Figure 1A). Meanwhile, *A. niger* produced

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174, 157, 105bp fragments (Figure 1B) and *A. terreus* yielded 4 fragments of 68, 105, 111, 161bp in size (Figure 1C). Though *Aspergillus* species restriction yielded characteristic fragments, however, no distinction between the isolates within each *Aspergillus* species.

β-tubulin genes sequence analysis

The phylogenetic analysis of the *Ben*A gene of *Aspergillus* species obtained in this study showed that they clustered separately into *A. flavus*, *A. niger* and *A. terreus* strains. Isolated strains from broiler breeder chickens and their hatching chicks are clustered with each other and with other strains available on the gene bank. However, 2 *A. flavus* and the *A. terreus* isolates from broiler breeder chickens showed a distinct phylogenetic relationship (Figure 2).

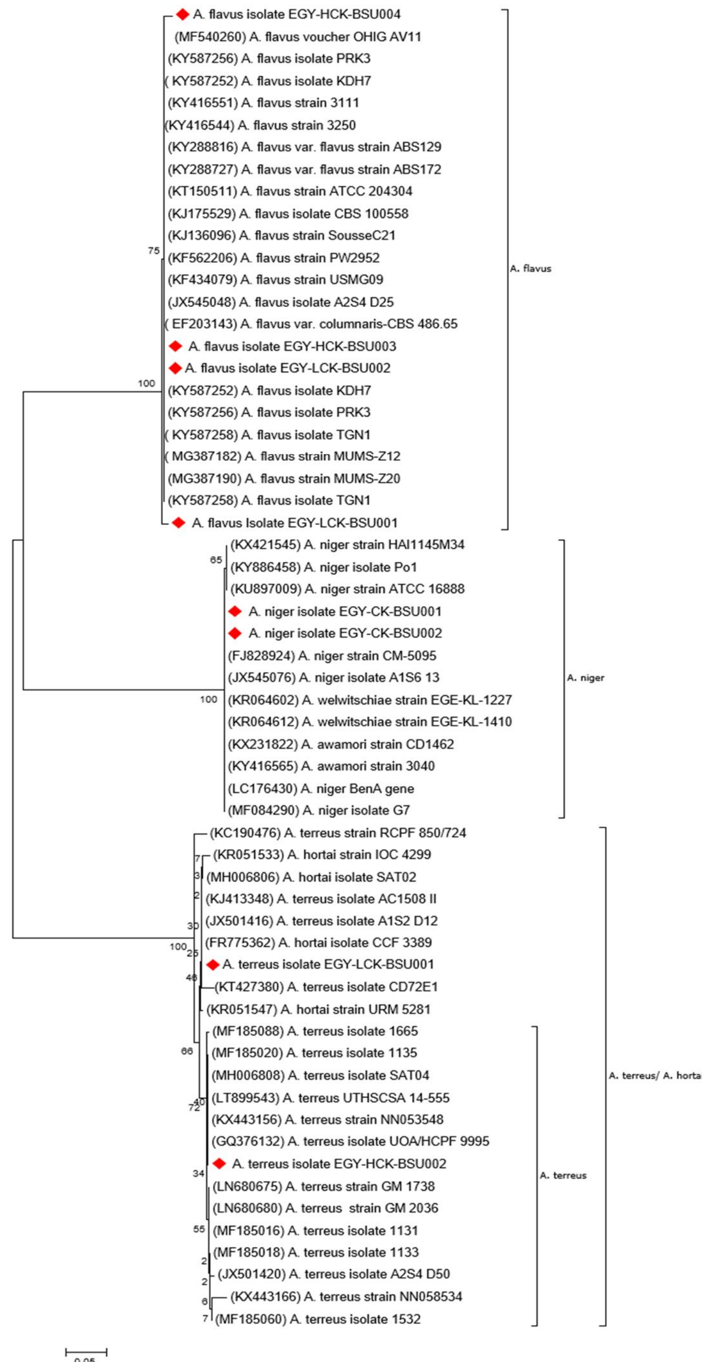

Figure 2. Phylogenetic analysis of partial ß-tubulin gene sequence of isolated Aspergillus spp. (red rectangle). Abbreviations: EGY, Egypt; LCK, breeder chicken; HCK, hatched chicks).

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Based on nucleotide sequence analysis *A. flavus* isolates EGY-LCK-002 obtained from breeder chickens is closely related to EGY-HCK-003 obtained from hatching chicks (100% identity). These 2 isolates are closely related to *A. flavus*- isolate MUMS-Z20 (MG387190.1) isolated from human patients suspected of pulmonary aspergillosis. The other 2 isolates are closely related to *A. flavus* isolates isolated from avian species and their feedstuff with nucleotide identities of 99.8% (Table 4, Figure 2).

Table 4. Nucleotide identity and number of nucleotide differences in the sequences of β-tubulin genes of *Aspergillus flavus* isolated from breeder chickens and their chicks

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	
1. *A. flavus* - EGY-LCK-BSU001		99.1	99.1	98.1	97.7	97.7	97.7	97.7	97.7	97.7	97.7	97.7	Nucleotide identities (%)
2. *A. flavus* - EGY-LCK-BSU002	3		100	99.1	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	
3. *A. flavus* - EGY-HCK-BSU003	3	0		99.1	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	
4. *A. flavus* - EGY-HCK-BSU004	6	3	3		99.1	99.1	99.1	99.1	99.1	99.1	99.1	99.1	
5. (MG387190) *A. flavus*- MUMS-Z20	8	1	1	3		100	100	100	100	100	100	100	
6. (KF434079) *A. flavus* - USMG09	8	1	1	3	0		100	100	100	100	100	100	
7. (KJ175529) *A. flavus* - CBS 100558	8	1	1	3	0	0		100	100	100	100	100	
8. (KJ136096) *A. flavus* - SousseC21	8	1	1	3	0	0	0		100	100	100	100	
9. (KY416551) *A. flavus* strain 3111	8	1	1	3	0	0	0	0		100	100	100	
10. (KY587258) *A. flavus* - TGN1	8	1	1	3	0	0	0	0	0		100	100	
11. (KY416544) *A. flavus* - 3250	8	1	1	3	0	0	0	0	0	0		100	
12. (KT150511) *A. flavus*-ATCC 204304	8	1	1	3	0	0	0	0	0	0	0		
	No. of differences												

The breeder chicken isolate *A. terreus* EGY-LCK-BSU001 showed 99.8% identity to *A. hortai* isolate SAT02 (MH006806.1) which is isolated from human bronchopulmonary disease cases. While the isolate *A. terreus* EGY-HCK-BSU001 from hatching chicks showed 99.5% identity to *A. terreus* isolate 1665 (MF185088.1) isolated from human clinical cases with *A. terreus* characterized by the intrinsic resistance to amphotericin B. The identity between the 2 obtained isolates is 96.3% (Table 5 and Figure 2). The 2 *A. niger* isolates obtained from broiler breeder chicken and their hatching chicks showed 98.9% identity between each other and 99.1-99.8% identity to other *A. niger* isolates of avian origin (data not shown).

Table 5. Nucleotide identity and number of nucleotide differences in the sequences of β-tubulin genes of *Aspergillus terreus* isolated from breeder chickens and their chicks

Isolate	1	2	3	4	5	6	7	8	9	10	
1. *A. terreus* isolate EGY-LCK-BSU001		96.3	99.5	98.5	95.9	99.5	95.8	99.5	96.6	95.9	Nucleotide identities %
2. *A. terreus* isolate EGY-HCK-BSU002	16		96.3	95.1	99.1	96	99.1	96	93.3	99.3	
3. (MH006806) *A. hortai* isolate SAT02	2	16		98.8	96.3	99.6	96.3	100	97	96.2	
4. (KR051533) *A. hortai* strain IOC 4299	7	23	6		95.9	98.9	95.6	99.1	96.6	95.7	
5. (MF185060) *A. terreus* isolate 1532	18	4	17	21		96.2	100	96.7	94.4	99.6	
6. (JX501416) *A. terreus* isolate A1S2 D12	2	18	2	6	19		96.6	99	96.3	95.9	
7. (MF185016) *A. terreus* isolate 1131	18	4	16	21	0	16		96.6	94	99.6	
8. (KJ413348) *A. terreus* isolate AC1508 II	2	18	0	5	16	5	16		97.5	96.7	
9. (KT427380) *A. terreus* isolate CD72E1	15	31	14	18	29	20	29	13		94.1	
10. (MF185088) A. terreus isolate 1665	17	3	16	20	2	20	2	16	29		
	No. of differences										

Discussion

The aim of this study was to investigate the prevalence and to genetically characterize isolated fungi agents in local Egyptian broiler breeder chickens and their hatching eggs. In broiler breeder chicken farms, the highest prevalence of fungal isolates was reported in tracheal swabs collected from breeder chickens in all breeds except Sainaa and Dahaby breeds that showed higher isolation rates from cloacal

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swabs, respectively. The nature of the *Aspergillus* conidia of being small in diameter (2-3μm) allows them to escape the respiratory tract barriers (Reese *et al.*, 2006). This may explain the high isolation rate from the respiratory tract of breeder chickens and hatched chicks. The feed is an important source for aflatoxin-producing fungi of the genus *Aspergillus*, 3 out of 8 feed samples collected were positive for fungal isolation. The 3 samples were restricted to Sainaa and Dahaby breeds. Notably, these three breeds showed the highest rate of isolation from cloacal swabs suggesting the possible contamination of feed via fecal matter prior to sample collections. Similar results were observed in the water samples collected.

Recent studies reported the contamination of the eggs with *Aspergillus* species with relatively high rates of 14% (Neamatallah *et al.*, 2009) and even up to 66% (Ahmed, 2015). Swab samples collected from incubators that incubating the eggs of Dokki and Sainaa breeds showed 50% fungal isolation rate (2 out of 4 samples). The positive samples yielded the isolation of only *Zygomycetes*. The low number of samples and the difficulty to collect samples in a timely manner make it difficult to judge the hygienic conditions of the hatcheries over the eggs incubation period. Higher isolation rates (80-100%) of fungi were observed in both dead-in-shell eggs and hatched chicks of Dokki and Sainaa breeds. In contrast, the Dahaby and Gemeza breed's dead-in-shell eggs and hatched chick's organ samples showed lower rates. The ability of filamentous fungi, especially those of the genus *Aspergillus*, to penetrate the eggshell during storage (Moustafa, 1995) and the suitability of embryonated eggs for *Aspergillus* species growth has been reported (Jacobsen *et al.*, 2010; Jacobsen *et al.*, 2012). Moreover, the high fungal isolation rates from dead-in-shell are relatively associated with high isolation rate in hatched chick indicating the important role played by the hatchery environment in the spread of fungal contaminants (Smith and Rehberger, 2018).

The *Aspergillus* species are known to favorably grow in poultry houses producing large amounts of spores (Kunkle, 2003). Generally, *A. fumigatus* and *A. flavus* are the most frequently isolated species from animal, avian and human infections and both are the etiological agent of Aspergillosis (Krishnan *et al.*, 2005; Pasqualotto, 2009). In this study, the species of the genus *Aspergillus* remained the most isolated fungi in breeder chickens and their hatched chick's samples collected. Though the morphotyping of Aspergilli enabled us to characterize the different isolated Aspergilli, they are of low value for identification on the level of the strains. Thus, various molecular approaches have been used for the identification of *Aspergillus* species (Brookman and Denning, 2000). The β-tubulin gene is involved in both vegetative growth and asexual sporulation of the *Aspergillus* spp. (May *et al.*, 1987; Geiser *et al.*, 2000). Therefore, the β-tubulin gene was chosen for further molecular characterization of the isolated fungi.

The PCR-RFLP of the most predominant 3 fungal species *A. flavus*, *A. niger*, and *A. terreus* from breeder chickens and their hatched chicks was conducted. The *BccI* restriction digestion consistently produced a characteristic pattern for each *Aspergillus* species. These results indicate that PCR-RFLP can be used to discriminate between *Aspergillus* species (Staab *et al.*, 2009). However, the method is unable to distinguish between each species strains. The β-tubulin gene is a slowly evolving, conserved gene with a high degree of interspecies variability made it reproducibly used for genetic studies of *Aspergillus* and related species (Geiser *et al.*, 1998; Balajee *et al.*, 2006; Peterson, 2008). Phylogenetic analysis of the β-tubulin gene of the selected *Aspergillus* species showed that they clustered separately with their corresponding strains from the GenBank.

Two *A. flavus* and *A. terreus* isolates from breeder chickens and their hatching chicks showed a slightly distinct phylogenetic relationship. Interestingly, nucleotide sequence analysis of these isolates revealed that *A. flavus* isolates; EGY-LCK-002 (breeder chickens) and EGY-HCK-003 (hatched chicks) showed their high identity to *A. flavus*-MUMS-Z20 (MG387190.1) isolated from human cases of pulmonary aspergillosis in the Middle East (Zanganeh *et al.*, 2018). In addition, the breeder chicken isolate *A. terreus* EGY-LCK-BSU001 is closely related to *A. hortai*-SAT02 recovered from human bronchopulmonary cases (Imbert *et al.*, 2018). The chick isolate *A. terreus* EGY-HCK-BSU001 was also related to an amphotericin B resistant *A. terreus*-1665 (MF185088.1) isolated from human clinical cases (Vaezi *et al.*, 2018). This finding is remarkable in the light of previous studies showing that *A. flavus* is the

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principal species involved in the invasive pulmonary aspergillosis and other infections in human in the Middle East, Africa, and southwest Asian countries (Hedayati *et al*., 2007; Khairallah *et al*., 1992; Taj-Aldeen *et al*., 2003). Additionally, the increase of *A. flavus* and *A. terreus* isolation rate in dead-in-shell eggs and hatched chicks (≥12%) compared to their breeder chickens indicate the potential exposure of poultry to a human type rather than avian type Aspergilli. The limitation of the absence of human samples from poultry farms and hatchery workers in the investigated premises restricted our ability to explore a potential zoonotic relationship of the isolated fungi.

Conclusion

The current study further confirmed that the *Aspergillus* species remain the most prevalent fungi in breeder chickens and hatcheries. The molecular characterization using PCR-RFLP method is an easy timesaving method for discrimination between the Aspergillus species; however, on the level of strain identification additional techniques such as the β-tubulin sequencing method are required. Finally, further epidemiological studies using molecular techniques to identify avian and human Aspergilli are needed to monitor zoonotic important Aspergilli especially those showing antifungal drug resistance properties.

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References

Abd El-Galil Y, El-Kenawy AI, El-Gmiey SR & Abd El-Latif MM. 1995. Bacterial causes of lowering hatchability and early embryonic deaths in balady hatcheries in Dakahlia Governorate. Assiut Veterinary Medical Journal, 33: 199-206.

Ahmed RSA. 2015. Characterization and differentiation of fungal and bacterial isolates associated with dead-in-shell embryos in breeder layers. Master thesis. Bacteriology, Mycology and Immunology Department, Beni-Suef University.

Akan M, Haziroğlu R, Ilhan Z, Sareyyüpoğlu B & Tunca R. 2002. A case of aspergillosis in a broiler breeder flock. Avian Disease, 46: 497-501. DOI: 10.1637/0005-2086(2002)046[0497: ACOAIA] 2.0.CO; 2

Altschul SF, Gertz EM, Agarwala R, Schaffer AA & Yu YK. 2009. PSI-BLAST pseudocounts and the minimum description length principle. Nucleic Acids Research, 37: 815-824. DOI: 10.1093/nar/gkn981

Arné P, Thierry S, Wang D, Deville M, Le Loc'h G, Desoutter A, Féménia F, Nieguitsila A, Huang W, Chermette R & Guillot J. 2011. Aspergillus fumigatus in Poultry. International Journal of Microbiology, 2011. DOI: 10.1155/2011/746356

Balajee SA, Nickle D, Varga J & Marr KA. 2006. Molecular studies reveal frequent misidentification of *Aspergillus fumigatus* by morphotyping. Eukaryot Cell 5: 1705-1712. DOI: 10.1128%2FEC.00162-06

Beernaert LA, Pasmans F, Van Waeyenberghe L, Haesebrouck F & Martel A. 2010. Aspergillus infections in birds: a review. Avian Pathology, 39: 325-331. DOI: 10.1080/03079457.2010.506210.

Brookman JL & Denning DW. 2000. Molecular genetics in Aspergillus fumigatus. Curr Opin Microbiol 3:468-474. DOI: 10.1016/S1369-5274(00)00124-7.

Cruickshank R, Duguid JP, Marmion BP & Swain RHA. 1975. Medical Microbiology, 12th Edition ed. London: Churchill. Livingstone.

Geiser DM, Dorner JW, Horn BW & Taylor JW. 2000. The phylogenetics of mycotoxin and sclerotium production in Aspergillus flavus and Aspergillus oryzae. Fungal Genetics and Biology, 31: 169-179. DOI: 10.1006/fgbi. 2000.1215.

Geiser DM, Frisvad JC & Taylor JW. 1998. Evolutionary relationships in Aspergillus section Fumigati inferred from partial β-tubulin and hydrophobin DNA sequences. Mycologia, 90: 831-845. DOI: 10.2307/3761325.

Hedayati MT, Pasqualotto AC, Warn PA, Bowyer P & Denning DW. 2007. Aspergillus flavus: human pathogen, allergen and mycotoxin producer. Microbiology, 153: 1677-1692. DOI: 10.1099/mic.0.2007/007641-0.

www.SID.ir

Archive of SID

Imbert S, Normand AC, Ranque S, Costa JM, Guitard J, Accoceberry I, Bonnal C, Fekkar A, Bourgeois N, Houze S, Hennequin C, Piarroux R, Dannaoui E & Botterel F. 2018. Species identification and in vitro-antifungal susceptibility of Aspergillus terreus species complex clinical isolates: a French multicentre study. Antimicrobial agents and chemotherapy. DOI: 10.1128/AAC.02315-17.

Jacobsen ID, Grosse K & Hube B. 2012. Embryonated chicken eggs as alternative infection model for pathogenic fungi. Methods in Molecular Biology, 845: 487-496. DOI: 10.1007/978-1-61779-539-8_34.

Jacobsen ID, Grosse K, Slesiona S, Hube B, Berndt A & Brock M. 2010. Embryonated eggs as an alternative infection model to investigate Aspergillus fumigatus virulence. Infection and Immunity, 78: 2995-3006. DOI: 10.1128/IAI.00268-10.

Khairallah SH, Byrne KA & Tabbara KF. 1992. Fungal keratitis in Saudi Arabia. Documenta ophthalmologica Advances in ophthalmology, 79: 269-276. DOI: 10.1007/BF00158257.

Krishnan S, Manavathu EK & Chandrasekar PH. 2005. A comparative study of fungicidal activities of voriconazole and amphotericin B against hyphae of Aspergillus fumigatus. Journal of Antimicrobial Chemotherapy, 55: 914-920. DOI: 10.1093/jac/dki100.

Kunkle RA. 2003. Aspergillosis. In: Saif YM, Editor. Diseases of Poultry, 11th Ed. Ames, Iowa, USA: Iowa State University press. Pages, 883-895.

Martin MP, Bouck KP, Helm J, Dykstra MJ, Wages DP & Barnes HJ. 2007. Disseminated Aspergillus flavus infection in broiler breeder pullets. Avian Disease, 51: 626-631. DOI: 10.1637/0005-2086(2007)51[626:DAFIIB]2.0.CO;2

May GS, Tsang ML, Smith H, Fidel S & Morris NR. 1987. Aspergillus nidulans beta-tubulin genes are unusually divergent. Gene, 55: 231-243. DOI: 10.1016/0378-1119(87)90283-6.

Moustafa GZ. 1995. Effect of hatchery sanitation on the hatchability of breeder eggs. Master thesis. Veterinary Hygiene Department, Faculty of Veterinary Medicine, Cairo University Egypt.

Neamatallah A, El-Leboudy A, Amer A & El-Shenawy N. 2009. Biosafety Against Fungal Contamination of Hen's Eggs and Mycotoxins Producing Species. Meteorology, Environment and Arid Land Agriculture Sciences, 20: 63-73.

Pasqualotto AC. 2009. Differences in pathogenicity and clinical syndromes due to Aspergillus fumigatus and Aspergillus flavus. Medical mycology, 47: S261-270. DOI: 10.1080/13693780802247702.

Peterson SW. 2008. Phylogenetic analysis of Aspergillus species using DNA sequences from four loci. Mycologia, 100: 205-226. DOI: 10.1080/15572536.2008.11832477.

Reese S, Dalamani G & Kaspers B. 2006. The avian lung-associated immune system: a review. Veterinary research, 37: 311-324. DOI: 10.1051/vetres:2006003.

Rippon JW. 1988. Medical Mycology-The Pathogenic Fungi and the Pathogenic Actinomycetes 3rd Edition. W.B. Saunders Co., Philadelphia, USA.

Smith AH & Rehberger TG. 2018. Bacteria and fungi in day-old turkeys vary among companies, collection periods, and breeder flocks. Poultry Science, 97: 1400-1411. DOI: 10.3382/ps/pex429.

Staab JF, Balajee SA & Marr KA. 2009. Aspergillus section Fumigati typing by PCR-restriction fragment polymorphism. Journal of clinical microbiology, 47: 2079-2083. DOI: 10.1128/jcm.00551-09.

Stoute ST, Bickford AA, Walker RL & Charlton BR. 2009. Mycotic pododermatitis and mycotic pneumonia in commercial turkey poults in northern California. Journal of veterinary diagnostic investigation, 21: 554-557. DOI: 10.1177/104063870902100424.

Taj-Aldeen SJ, Hilal AA & Chong-Lopez A. 2003. Allergic Aspergillus flavus rhinosinusitis: a case report from Qatar. European archives of oto-rhino-laryngology, 260: 331-335. DOI: 10.1007/s00405-002-0547-x.

Tamura K, Stecher G, Peterson D, Filipski A & Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular biology and evolution, 30: 2725-2729. DOI: 10.1093/molbev/mst197.

Vaezi A, Fakhim H, Arastehfar A, Shokohi T, Hedayati MT, Khodavaisy S, Rezaei-Matehkolaei A, Badiee P, Hagen F, Lass-Florl C, Dannaoui E, Meis JF & Badali H. 2018. In vitro antifungal activity of amphotericin B and 11 comparators against Aspergillus terreus species complex. Mycoses, 61: 134-142. DOI: 10.1111/myc.12716.

www.SID.ir

Archive of SID

Wang DY. 2012. Genetic diversity and antifungal susceptibility of Aspergillus spp. isolates from avian farms in Guangxi, China. Ph.D. thesis. Agricultural sciences, AgroParisTech, The National veterinary school of Alfort. Paris, France.

Zanganeh E, Zarrinfar H, Rezaeetalab F, Fata A, Tohidi M, Najafzadeh MJ, Alizadeh M & Seyedmousavi S. 2018. Predominance of non-fumigatus Aspergillus species among patients suspected to pulmonary aspergillosis in a tropical and subtropical region of the Middle East. Microbial pathogenesis, 116: 296-300. DOI: 10.1016/j.micpath.2018.01.047

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