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Original Research Article

Fungal pathogens associated with respiratory problems in broiler chickens

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ABSTRACT

Fungal diseases of poultry have become problematic as bacterial and viral diseases. Therefore, this study was designed to investigate the prevalence of fungal agents in broiler chickens suffering from respiratory disorders. The prevalence rate of fungal isolation was 53.1% including mycelia fungi (42%) and yeast isolates (11.1%). Mould isolates were identified as *Aspergillus fumigatus* (21.7%), *Aspergillus flavus* (8.4%), *Aspergillus niger* (8.4%), *Aspergillus nidulans* (1.3%), *Cladosporium* spp. (0.4%) and *Penicillium* spp. (1.8%). Concerning yeast isolates, *Candida* spp. was the most predominant which were identified as *C. albicans*, *C. pseudotropicalis* (2.7% each), *C. krusei*, *C. regosa* (2.2% each), and *C. stellatoidea* (0.4%). Moreover, one Cryptococcus sp. was recovered and identified as *C. neoformans* (0.9%). PCR assay using oligonucleotide primer amplifying a 570 bp fragment based on ITS region gene was conducted on randomly selected 9 isolates including 6 *Aspergillus* spp. (*A. flavus*, *A. niger*, *A. fumigates*; 2 each) as well as 3 yeast isolates (two isolates of *C. albicans* and one isolate of *C. neoformans*). All tested isolates had positive reactions.

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1. Introduction

Globally, the poultry industry has shown impressive performance compared with other livestock enterprises. Despite its rapid growth, it is facing various constraints, of which infectious and non-infectious diseases/outbreaks considered as serious problems (Windhorst, 2006; Bhuvaneswari, 2008).

In Egypt, the poultry industry is one of the main agricultural industries, where investment in this industry is about 18 billion LE. The size of the labour force is approximately 1.5 million permanent workers and approximately one million temporary workers. The industry contributes a large section of the country's supply of animal protein (white meats and eggs) (El Nagar and Ibrahim, 2005).

Among fungi, *Penicillium* and *Aspergillus* species are dominantly present (Plewa-Tutaj and Lonc, 2014). Species of such genera are commonly found in soil, decaying organic materials, animal feed, stored grains and other materials (Leite Jr et al., 2012). Moreover, fungal species are responsible for spoilage of food and beverages, biodeterioration of materials producing dangerous mycotoxins.

There are common and important diseases affecting the respiratory system of poultry. Fungal infections are frequently associated with morbidity and mortality in birds. Few fungal species, particularly *Aspergillus*; the cause of aspergillosis, which is composed of approximately 600 species, are common avian pathogens (Rippon, 1982). Manifestations of aspergillosis depend upon organs or systems involved and whether infection is localized or disseminated. Aspergillosis in birds is usually confined to the lower pulmonary system with florid lesions in air sacs and lungs. Less common manifestations related to infections of the eye, brain, skin, joints, and viscera (Wallace, 1976). Aspergillosis appears to be more significant in confinement situations where stress factors may be involved or where mouldy litter or grain is present. Contaminated poultry litter is often the source of *Aspergillus* conidia (Dyar et al., 1984).

Acute aspergillosis often occurs in young birds resulting in high morbidity and mortality. The chronic form is sporadic. It causes lesser mortality and usually affects elder birds, particularly breeders, presenting a compromised immune system due to poor husbandry conditions (Kunkle et al., 2003; Redig, 2005). In spontaneous outbreaks, mortality ranged between 4.5% and 90%, whereas age of diseased birds varied from 3 days to 20 weeks (Martin et al., 2007). Moreover, feed conversion and growth rate in recovering birds remain poor. Indeed, airsacculitis is a major reason for carcass condemnation at slaughter inspection (Kunkle et al., 2003).

Aspergillus fumigatus is considered as a major respiratory pathogen in birds. Such filamentous fungus was first described in lungs of a bustard in 1863 by Fresenius (Castellani, 1928). Other species like *A. flavus*, *A. niger*, *A. nidulans*, and *A. terreus* were isolated from avian cases of aspergillosis (mixed infections are possible) but much less frequently than *A. fumigatus* (Kunkle et al., 2003; Martin et al., 2007). Active fungal proliferation and sporulation of *A. fumigatus* on organic materials produce large amounts of airborne small-sized conidia that are easily dispersed in the air, then potentially inhaled and deposited deep in the respiratory tract of broilers and so bronchopneumonia is developed (Milos et al., 2011).

Moreover, *Candida* species infection is widely spread throughout the poultry affecting several areas worldwide. Recently, the growing economic value of poultry has led to the increase of research of poultry diseases. Fungal diseases of poultry have become problematic as bacterial and viral diseases (Darwish, 1989).

The purpose of this study was to investigate the prevalence of fungal infection in broiler chickens as well as to identify the phenotypic characters of different fungal isolates.

2. Material and methods

2.1. Samples

A total of 226 samples were collected from broiler chickens of various ages suffering from respiratory disorders obtained from different localities in Beni-Suef and El-Fayoum governorates during the period from September 2013 to April 2014. Specimens from different lesions including air sacs (87), pericardium (68), kidneys (56) and liver (15) were taken and were directly transferred to the laboratory of Department of Microbiology, Faculty of Veterinary Medicine at Beni-Suef University for mycological examinations.

2.2. Fungal isolation

Samples were immediately transferred directly into pre-enrichment broth (Malt extract broth,

Oxoid) and incubated at 37°C for 24-48 h, then cultured on Sabouraud dextrose agar medium (Oxoid) and incubated at 37°C for 24-48 h.

2.3. Identification of fungal isolates

The recovered fungi were morphologically identified according to Rippon (1988). Mycelial fungi were identified by examination of mycelial morphology, the reverse colour as well as examination of lactophenol cotton blue stained smears. Yeast-like fungi were identified by colonial morphology and examination of Gram`s stained smears, then they were confirmed by further biochemical laboratory tests as sugar fermentation tests (glucose, maltose, galactose and lactose) and urease test (Rippon, 1988).

2.4. Germ tube test

All recovered yeast isolates underwent Gram tube test to identify *C. albicans* using human serum according to Rippon (1988).

2.5. Chlamydospore production

It was used to identify *C. albicans* using rice extract agar medium according to Taschdjian (1953). All recovered yeast isolates were inoculated onto rice agar by making 3 parallel cuts about 5 mm apart into agar (Rippon, 1988). A coverslip was laid on the surface of the agar covering a portion of inoculation streaks. Inoculated plates were incubated at 30˚C for 24-48 h and microscopically examined through the coverslip for the presence of hyphae, blastoconidia, chlamydoconidia or arthroconidia.

2.6. Polymerase chain reaction

PCR using oligonucleotide primers that amplify a 570 bp fragment in ITS1 gene of *Aspergillus* species and yeast (Mirhendi et al., 2007) was applied on 9 randomly selected isolates; 6 mould (*A. flavus*, *A. niger*, *A. fumigatus*; two each) and 3 yeast (2 isolates of *C. albicans* and one isolate of *C. neoformans*) isolates.

2.6.1. Primers

~ Primer 1 (forward primer):
5'- *TCCGTAGGTGAACCTGCG G* - 3'
~ Primer 2 (reverse primer):
5'- *TCCTCCGCTTATTGATATGC* - 3'

3. Results

3.1. The prevalence of fungal pathogens recovered from different internal lesions in broiler chickens suffering from respiratory disorders

Out of 226 samples collected from different lesions of broilers chicken with respiratory disorder, 120 fungal isolates were recovered with a prevalence rate of 53.1%, including 95 (42%) mould isolates and 25 (11.1%) yeast isolates. Forty four isolates (50.6%) were from air sacs of which 36 (41.4%) moulds and 8 (9.2%) yeasts were detected. Twenty nine (42.6%) isolates obtained from heart; among those 20 (29.4%) moulds and 9 (13.2%) yeasts were found. In kidneys, 42 (75%) isolates were recovered; 34 (60.7%) moulds and 8 (14.3%) yeasts; while 5 (33.3%) mould isolates were recovered from the liver with no yeasts detection (Table 1).

Table 1. The prevalence of fungal pathogens recovered from different internal lesions.

Organ	Number of samples	Mycelial fungi		Yeasts		Total	
		No.	%	No.	%	No.	%
Air sacs	87	36	41.4	8	9.2	44	50.6
Heart	68	20	29.4	9	13.2	29	42.6
Kidney	56	34	60.7	8	14.3	42	75
Liver	15	5	33.3	0	0	5	33.3
Total	226	95	42	25	11.1	120	53.1

No.: Number of positive samples %: Percentage according to No. of samples

3.2. Identification of recovered mould isolates

Mycological identification of 95 mould isolates recovered from 226 samples broiler chickens

revealed the following species; *Aspergillus fumigatus* (49; 21.7%), *Asperigllus flavus* (19; 8.4%), *Aspergillus niger* (19; 8.4%), *Aspergillus nidulans* (3; 1.3%), *Cladosporium* spp. (one ; 0.4%) and *Penicillium* spp. (4; 1.8%) (Table 2).

3.3. Identification of recovered yeast isolates

Out of 25 yeast isolates recovered from 226 broiler chickens, 23 *Candida* spp. and two *Cryptococcus* spp. were identified. *Candida* species were identified as *C. albicans* (6; 2.7%), *C. stellatoidea* (one; 0.4%), *C. pseudotropicalis* (6; 2.7%), *C. krusei* (5; 2.2%) and *C. rugosa* (5; 2.2%). The recovered *Cryptococcus* sp. was identified as *C. neoformans* (0.9%) (Table 3).

Table (2): Recovery rate of mould isolated from broiler chicken samples.

	Mould isolates													
Number of samples	*A.fumigatus*		*A. flavus*		*A. niger*		*A.nidulans*		*Cladosporium* spp.		*Pencillum* spp.		Total	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
226	49	21.7	19	8.4	19	8.4	3	1.3	1	0.4	4	1.8	95	42

No.: Number of positive samples %: Percentage according to total No. of samples.

Table 3. The recovery rate of mould isolated from broiler chicken samples.

Number of samples	*C. albicans*		*C. stellatoidea*		*C. pseudotropicalis*		*C. krusei*		*C. rugosa*		*C. neoformans*		**Total**	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
226	6	2.7	1	0.4	6	2.7	5	2.2	5	2.2	2	0.9	25	11.1

No.: Number of positive samples %: Percentage according to total No. of samples.

3.4. Chlamydospore production and Germ tube test

Among the tested yeasts (25 isolates), all *C. albicans* isolates (*n*=6) were the only producing chlamydospores and forming germ tube, while the rest of *Candida* species (*n*=17) as well as *Cryptococcus* species (*n*=2) did not (Figs. 1, 2).

3.5. Polymerase chain reaction

All 9 isolates (6 moluds and 3 yeasts) showed positive reaction with PCR test using oligonucleotide primer that amplified a 750 bp fragment in ITS1 gene (Fig. 3).

4. Discussion

Among the infectious diseases, fungal affections have their own importance and seem to be one of the great obstacles for the poultry farmers. Species of the genus *Aspergillus* are important fungal infection, affecting the respiratory tract of birds causing high morbidity, mortality and production losses (Richard et al., 1991). Moreover, mycological examination to investigate mycotic flora of chicken population revealed an isolation of fungal isolates such as *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus flavus* and *Mucor* indicating the ubiquitous nature of these fungi (El-Badry and Sokkar, 1988).

Moreover, *Candida* species are widely spread throughout the poultry producing districts worldwide. Poultry of all ages are susceptible to the effects of such organism. Chickens, turkeys, pigeons, pheasants, quails and grouse are the most commonly affected species. Recently, the growing economic value of poultry led to the increase of research of poultry diseases. Fungal diseases of poultry have become problematic as bacterial and viral diseases (Darwish, 1989).

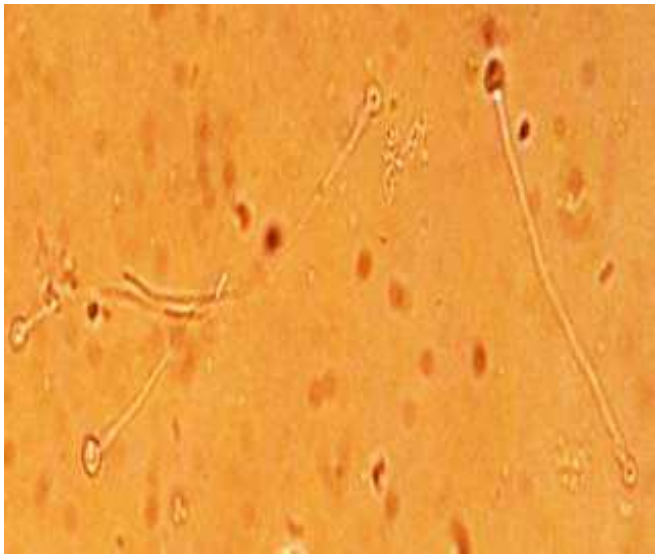

Fig. 1. Light microscopy of germ tube formation of *C. albicans*. Germ tubes appear as hypha-like extensions of yeast cells, produced usually without a constriction at the point of origin from the cell.

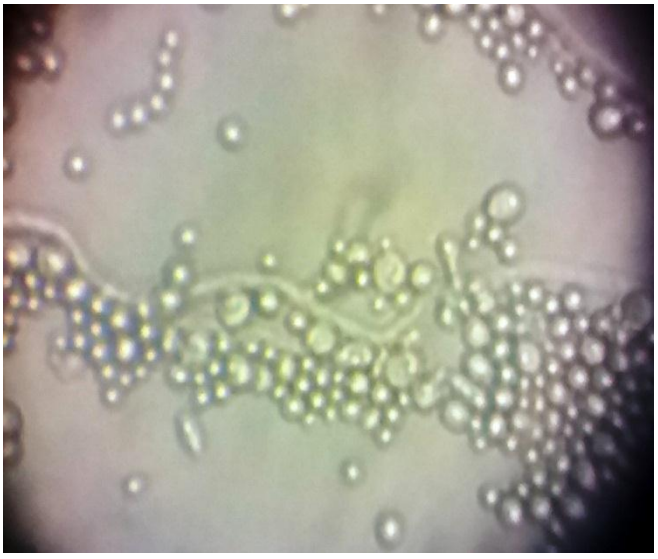

Fig. 2. Light microscopy of Chlamydospore production on rice agar medium. Yeast cells appear as spherical clusters at regular intervals on pseudohyphae and having the chlamydospores on pseudohyphae-like shiny pearls.

In the present study, the prevalence rate of fungal isolation from broiler chickens was 53.1% including mycelial fungi (42%) and yeast isolates (11.1%) (Table 1). Such finding was higher than that of Radwan et al. (2014) who reported that the prevalence rate of fungal isolation from broiler chickens was 39%. Moreover, they recorded that 36% of fungal isolates were yeast and 3% were mycelial fungi, opposite to those obtained in the present study. These results were more or less similar to those of Pennycott et al. (2003) who isolated yeast from chicken's samples in a percentage of 12.15%. On the other hand, the present prevalence was lower than that obtained by Abd El-Aziz (2015) who reported that the prevalence rate of fungal isolation from chickens was 66.9%.

In the current work, the mycological identification of the mould isolates revealed 21.7% *Aspergillus fumigatus*, 8.4% *Aspergillus flavus*, 8.4% *Aspergillus niger*, 1.3% *Aspergillus nidulans*, 0.4% *Cladosporium* spp., and 1.8% *Penicillium* spp. (Table 2). Such findings run parallel to those obtained by Abd El-Aziz (2015) who reported that *A. fumigatus* represented 28.0%, *A. niger* 18.6%, *Cladosporium* spp. 11.9% and *Penicillium* spp. 8.5%. Similarly, the presence of the aforementioned fungal species was described from poultry farm by El-Zarka (1988) who reported the presence of *A. niger*, *A. fumigatus*, *Penicillium* spp. and *Cladosporium* spp., and *A. flavus*. Comparable results were stated where fungal isolates represented about 41.7% of poultry samples and *A. niger*, *A. fumigatus* represented the majority of such isolates (12.5% and 10%, respectively) (El-Badry and Sokkar, 1988).

Moustafa (1995) found that 35.2% of recovered isolates were *A. flavus*, 27.5% *A. niger*, 23.1% *A. fumigatus* and 14.3% *A. terreus*. However, there was neither *Cladosporium* nor *Penicillium* spp. The most predominant recovered fungi were *A. fumigatus* followed by A. *flavus* and *A. niger*. Such results supported findings reported by Richard et al. (1991).

Moreover, Radwan et al. (2014) found that all mycelial isolates recovered from broiler chickens belonged to *A. niger* and *A. flavus*.

Identification of the recovered yeast isolates from broiler chickens revealed *Candida* spp. were the most prevalent and were identified as *C. albicans* and *C. pseudotropicalis* (2.7% each), *C. krusei* and *C. rugosa* (2.2% each) then *C. stellatoidea* (0.4%).

Moreover, one *Cryptococcus* sp. was recovered and identified as *C. neoformans* (0.9%). Such results run hand to hand with those of Radwan et al. (2014) who found that *C. albicans* was the most prevalent (19%) followed by *C. pseudotropicalis* (6%), *C. krusei* (5%) and *C. rugosa* (4%). They also recovered 2 isolates of *Cryptococcus* spp. identified as *C. neoformans* and *C. laurentii*. Moreover, Wyatt and Hamilton (1975) found that *C. albicans* comprised 95% of isolates, while *C. ravautii*, *C. salmonicola*, *C. gulliermondi*, *C. papapsilosis*, *C. catenulata* and *C. brumptii* comprised the remainder.

Chlamydospore production is considered the most important diagnostic feature in the laboratory identification of *Candida albicans* (Benham, 1931). Corn meal infusion agar has been most satisfactory for this purpose but certain inconveniences in the preparation of this medium led Taschdjian (1953) to develop a rice extract agar for chlamydospore production. The medium is relatively simple to prepare from readily available materials and its value in the stimulation of chlamydospore production has been confirmed by Sina and Reiss (1975).

The germ tube test provides a simple, reliable and economic procedure for the presumptive identification of *C. albicans*. Approximately 95% of clinical isolates produce germ tubes when incubated in serum at 35˚C for 2.5 to 3 h. A germ tube represents the initiation of a hypha directly from the yeast cell. They have parallel walls at their point of origin. Germ tube formation is influenced by the medium, inoculum size and temperature of incubation. Fresh normal pooled human sera or a commercially available germ tube solution (Remel Lenexa kansa) are to be used as the medium for the test. The inoculum should result in a very faintly turbid serum suspension. Over inoculation inhibits the development of germ tubes (Alexopolous et al., 1996).

The present results showed that *C. albicans* isolates (n=6) were the only forming germ tube and producing chlamydospores while the rest of *Candida* spp. (n=17) as well as *C. neoformans* (n=2) did not, and both tests were considered additional confirming laboratory tests for the presumptive identification. These results were similar to those obtained by Radwan et al. (2014).

Though the morphological and microscopic identification of *Aspergillus* spp. has been well established, they are usually labor intensive and need expert mycologist to differentiate between fungal species. Therefore, various molecular approaches have been used for the identification of *Aspergillus* spp., including PCR amplification and fragment length analysis, DNA probe hybridization or sequence analysis (Hinrikson et al., 2005). Molecular techniques used for identification and characterization using the Internal Transcribed Spacer (ITS) region of *Aspergillus* spp. has been proven to be useful for genetic studies of *Aspergillus* spp. and related species, because it appears to be a slowly evolving, conserved gene with a high degree of interspecies variability (Mirhendi et al., 2007; Staab et al., 2009).

In the present study, randomly selected 6 *Aspergillus* isolates (*A. flavus*, *A. niger*, *A. fumigatus*; two isolates each) as well as 3 yeast isolates (two isolates of *C. albicans* and one isolate of *C. neoformans*) that have been identified morphologically and microscopically were further characterized using oligonucleotide primers targeting ITS region gene. It has been found that all 9 isolates showed positive reactions with PCR with amplification of the 570 bp fragment of ITS of *Aspergillus* spp. and yeasts.

Results further confirm the conclusion stated by Kanbe et al. (2002) indicating that ITS gene PCR method is rapid, simple and available as a tool for identification of *Aspergillus* spp. Furthermore, the molecular characterization of the ITS region could be used as a target for molecular identification of *Aspergillus* spp. and yeasts pathogenic for poultry with the same efficiency of identification of medically important species in human medicine such as *A. flavus, A. granulosus,* and *A. nidulans* as well as *C. albicans* (Hinrikson et al., 2005).

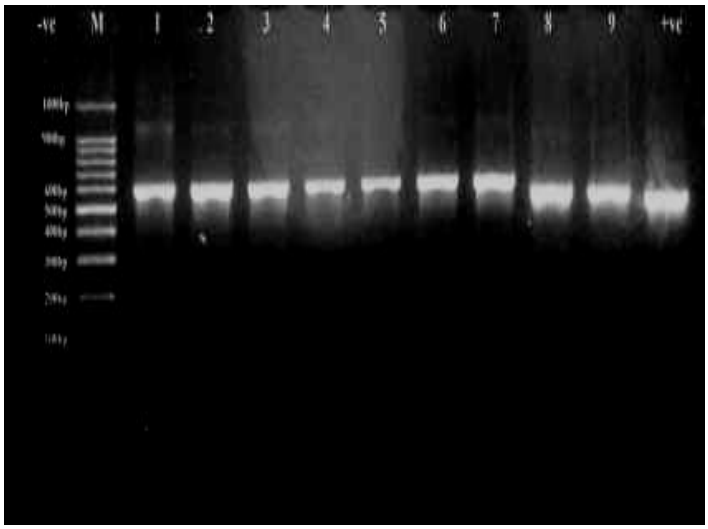

Fig. 3. PCR findings for 9 fungal isolates. Amplification of the 570 bp fragment of ITS1 gene from tested isolates showed a positive amplicon migrates with molecular size of about 570 bp using the molecular DNA size marker. M: 100bp ladder. +ve: Control positive (*A. niger*). Lanes (1-2) *A. flavus*, (3-4) *A. niger*, (5-6) *A. fumigatus*, (7-8) *C. albicans*, (9) *C. neoformans*

5. Conclusion

Fungal diseases of poultry have become problematic as bacterial and viral diseases. The prevalence rate of fungal infection in examined broiler chickens was 53.1% including 42% mycelia fungi and 11.1% yeast. *Aspergillus fumigatus* was the most prevalent isolate.

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