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Fungi Associated with Dead-in-Shell Embryos of Chicken and Turkey Layers

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DEAD-IN-SHELL embryos is represented the main cause of hatchery losses in poultry. There is limited literature about the role of fungi in the hatchability problems. In this study, different fungi were isolated from dead-in-shell embryos of local Egyptian breeds. Some randomly selected isolates were molecularly identified, and the antifungal activity of extracted essential oils was investigated on these isolates. Seventy nine fungal isolates were recovered in the rate of 66.9% of which *A. fumigatus*, *A. niger*, *Cladosporium* spp. and *Penicillium* Spp. were represented in a prevalence of 28.0, 18.6, 11.9 and 8.5% respectively. *Aspergillus* spp. remains the highest prevalent fungi that recovered from dead-in-shell embryos among chicken and Turkey embryos. The cinnamon and thyme oil are the superior antifungals extract with concentrations as low as 0.05%, followed by clove oil. However, high concentrations of rosemary (up to 0.5%) are needed to inhibit the fungal growth. Fungal isolates were successfully characterized using PCR employing specific primers for the ITS region and β-tubulin gene of *Aspergillus* spp. In conclusion, these results indicated the importance of *Aspergillus* spp. in hatchability problems. PCR detection was proven to be the more rapid and the simplest tool for identification of *Aspergillus* spp. Moreover, essential oils can be used as efficient antifungals.

Keywords: *Aspergillus* spp., Essential oils, β-tubulin gene, ITS region

Dead-in-shell chicken embryos constitutes one of the several factors accounting for lowered hatchability of incubated eggs (Orajaka and Mohan, 1985).

Despite frequent evidences of the ability of the fungi and/or their byproducts to invade the embryonated eggs and causing embryo mortality, there is very limited literature available on their exact role in the hatchability problems. For instance, mycotoxins, by-products of fungal metabolism, have been implicated as causative agents of adverse health effects in birds (Qureshi *et al.*, 1998 and Brake *et al.*, 1999). Fungi, primarily *Aspergillus* (*A.*) *fumigatus*, proliferated on the air cell membrane in some of the un-hatched eggs, and it was suggested that the hole provides a means for the fungal conidia, to be introduced to the air cell area during the hatching period (Williams *et al.*, 2000).

More evidences supported with the field observations during the necropsy of un-hatched broiler eggs following egg injection indicated the ability of fungi to proliferate effectively in embryonated eggs. The subsequent contamination of the early hatched chicks with fungi was proposed as these infertile and early dead embryos due to fungal growth may serve as a reservoir for infection (Williams and Brake, 2000). The embryonated eggs were suggested as an alternative infection model to investigate *A. fumigatus* virulence as compared to mouse model. The fungus caused embryonic death by invasion of embryonic membranes and blood vessels. The ability of Asperagillus fungus to grow in eggs indicates the probable fungal invasion to embryonated eggs with the potential of causing hatchability problems (Jacobsen *et al.*,2012). Such examples of the ability of both the fungi themselves and their by-products to cause embryo mortality have raised our concern to investigate the potential role of these organisms in the problem of reduced egg hatchability in the poultry.

The identification of *Aspergillus* spp. usually based on morphological characters which requires adequate amount of growth for evaluation the colony characteristics and the microscopic examination (Henry *et al.,* 2000). The traditional identification procedures are frequently labor intensive and need expert mycologist to differentiate between fungal species. Due to these limitations, several molecular methods have been used for the specific identification of *Aspergillus* spp., for instance PCR, fragment length analysis, DNA probe hybridization and sequence analysis (Bretagne *et al.,* 1995 and Hinrikson *et al.,* 2005).

The usages of plantectracts as disease control agents have been studied, ever since they have low toxic effect on mammalian tissue, less environmental effects and wide public acceptance (Lee *et al., 2*007). Therefore, there has been increasing attention to substitute synthetic preservatives with natural, effective and nontoxic compounds. Those are, in the first place, extracts and essential oils of spices and herbs (Smid and Gorris, 1999). Many essential oils and their ingredients possess antibacterial, antifungal, and antiviral properties (Soliman & Badeaa, 2002 and Tzortzakis, 2009). Their mechanism of action was suggested to cause decreased conidiation, leakage of cytoplasm, loss of pigmentation and disrupted cell structure indicating fungal wall degeneration (Carmo *et al.,* 2008).

With these facts in mind, this work was designed to isolate and characterize fungi associated with dead-in-shell embryos in chicken and turkey local breeds, molecular identification of some selected isolates using PCR, and to study the antifungal effect of some essential oils against selected isolates recovered from dead-in-shell embryos.

Material and Methods

Samples

A total of 118 samples were collected as dead-in-shell embryos chicken (No =96) and turkey (No=22) at different embryonic stages. The samples were obtained mainly from automatic hatcheries (El-Azab Integrative Poultry Project,

Fayoum governorate). The routine egg sanitation procedure in this hatchery included fumigation with formaldehyde gas in a concentration of 20 gm/m^3.

Isolation and identification of fungal agents

The egg shell was disinfected with 70% ethyl alcohol. A sufficient area of the shell was removed around the air sac with a pair of sterile scissors. A loop full from the yolk, embryonic fluids, liver, spleen and heart were used for fungal isolation. Samples from egg contents were inoculated onto Sabouraud's dextrose broth (SDB) and incubated for 3-5 days at 25-27°C. A loop full from selective broth was plated onto Sabouraud's dextrose agar (SDA) containing chloramphenicol 50 mg/liter and incubated for 3-5 days at 25-27°C.

Molecular detection of fungal isolates

Fungal DNA was extracted using 100 mg of fungal samples that were placed into liquid nitrogen and grind thoroughly with a mortar and pestle. Gene-JET Genomic extraction according to the manufacturer instructions. One primer pair targeting the internal transcribed spacer 1 (ITS) region (ITS-F, 5'-TCCGTAGGTGAACCTGCGG-3' and ITS-R, 5'-TCCTCCGCTTATTGATA TGC-3') (Mirhendi *et al.*, 2007) and the other primer targeting ß-Tubulin gene (ß-tubF, 5'-AATTGGTGCCGCTTTCTGG-3' and ß-tubR, 5'-AGTTGTCGGG ACGGAATAG-3') (Staab *et al.*, 2009) were used for PCR amplification.

The reaction was performed using Genaid® Ultrapure Taq Polymerase PCR master mix (Genaid, U.K.). A reaction volume of 50 µl was consisted of 10 µl of 10X PCR master mix, 1µl of 20 pmol primers, 5µl of DNA extract and the volume was completed to 50µl using sterile deionized water. The thermal profile was an initial denaturation at 94°C for 2min, followed by 35 cycles of denaturation at 94°C for 30s, annealing at 53°C for 45s and extension at 72°C for 45s then a final extension step at 72°C for 7min. Amplified products in both reactions were visualized by 1.5% agarose gel electrophoresis in TBE buffer, stained with 0.5mg/ml ethidium bromide solution, and photographed.

Agar dilution method for detection of antifungal activity of essential oils

The antifungal activity of thyme, clove, cinnamon and rosemary oils against 20 randomly selected fungal isolates were evaluated (*A. fumigatus*, *A. niger*, *Penicillium* spp. and *Cladosporium* spp., 5 each). The tested fungi were grown on SDA at 35°C for 48 hr.

The oil was tested for sterility after millipore filtration by introducing 2 ml of this supposed sterile extract into 10 ml of sterile nutrient broth. Incubation was done at 37°C for 24 h. Sterility was detected by absence of turbidity of the broth after the incubation period.

The agar dilution method was done according to (Jeff-Agboola *et al.*, 2012), Briefly a final concentration of 0.5% (v/v) Tween-20 (Sigma) was incorporated into the agar after autoclaving to enhance oil solubility. A concentration of 1%,

0.5, 0.25, 0.1 and 0.05 (v/v) were prepared in sabouraud dextrose agar (SDA) with 0.5% (v/v) Tween-20. Plates were dried at 35°C for 30 min prior to inoculation with 1–2 ml spots containing approximately 10 CFU of each organism. SDA, with 0.5% (v/v) Tween-20 without oil, was used as a positive growth control. Inoculated plates were incubated at 35°C for 48 h then they were examined daily for 8 days and the oil dilution at which the fugal growth inhibited was recorded for each isolate.

Results

Prevalence of fungal isolates in dead-in-shell embryos:

Mycological examination of 118 dead-in-shell embryo egg samples revealed 79 fungal isolates in a rate of 66.9% (Table 1), of which 4 different fungal species, *A. fumigatus* (no=33, 28%), *A. niger* (no=22, 18.6%), *Cladosporium spp*. (no=14, 11.9%) and *Penicillium spp*. (no=10, 8.5%) were isolated. The isolated *A. fumigatus* was rapidly growing mycelial fungi (2 to 4 days) which produced a fluffy to granular green colony with white periphery. Microscopically *A. fumigatus* showed septated hyphae and short or long conidiophores. The tip of the conidiophore expanded into a large, dome-shaped vesicle having bottle-shaped phialides (Fig. 1 A&B).

A. niger produced mature colonies within 2 to 4 days. Growth began initially as a white colony that soon developed a black, dotted surface as conidia were produced. Microscopically, *A. niger* exhibited septated hyphae, long conidiophores that supported spherical vesicles that gives rise to large metulae and smaller phialides from which long chains of brown rough-walled conidia were produced (Fig. 1 C&D). Colonies of *Penicillium spp.* had shades of green or blue green. The surface of these colonies was velvety to powdery. Microscopically, hyphae were hyaline and septated and produce broom like conidiophores. Conidiophores exhibited branching metulae from which phialides producing chains of raised conidia (Fig. 1 E&F). *Cladosporium spp.* showed slow growth. Colonies were grey or olive green color, velvety in texture. Microscopically, *Cladosporium spp.* has brown, olive to black hyphae and conidia.

TABLE 1. Prevalence of different fungal species isolated from dead-in-shell embryos

Fungal Spp.	Chicken (No=96)	Turkey (No=22)	Total (No=118)
A. fumigatus	28 (29.2%)	5 (22.7%)	33 (28.0%)
A. niger	20 (20.8%)	2 (9.09%)	22 (18.6%)
Cladosporium spp	14 (14.6%)	0 (0.0%)	14 (11.9%)
Penicillium spp	9 (9.4%)	1(4.56%)	10 (8.5%)

No: number of tested samples

%: percentage of positive samples in relation to the total tested number

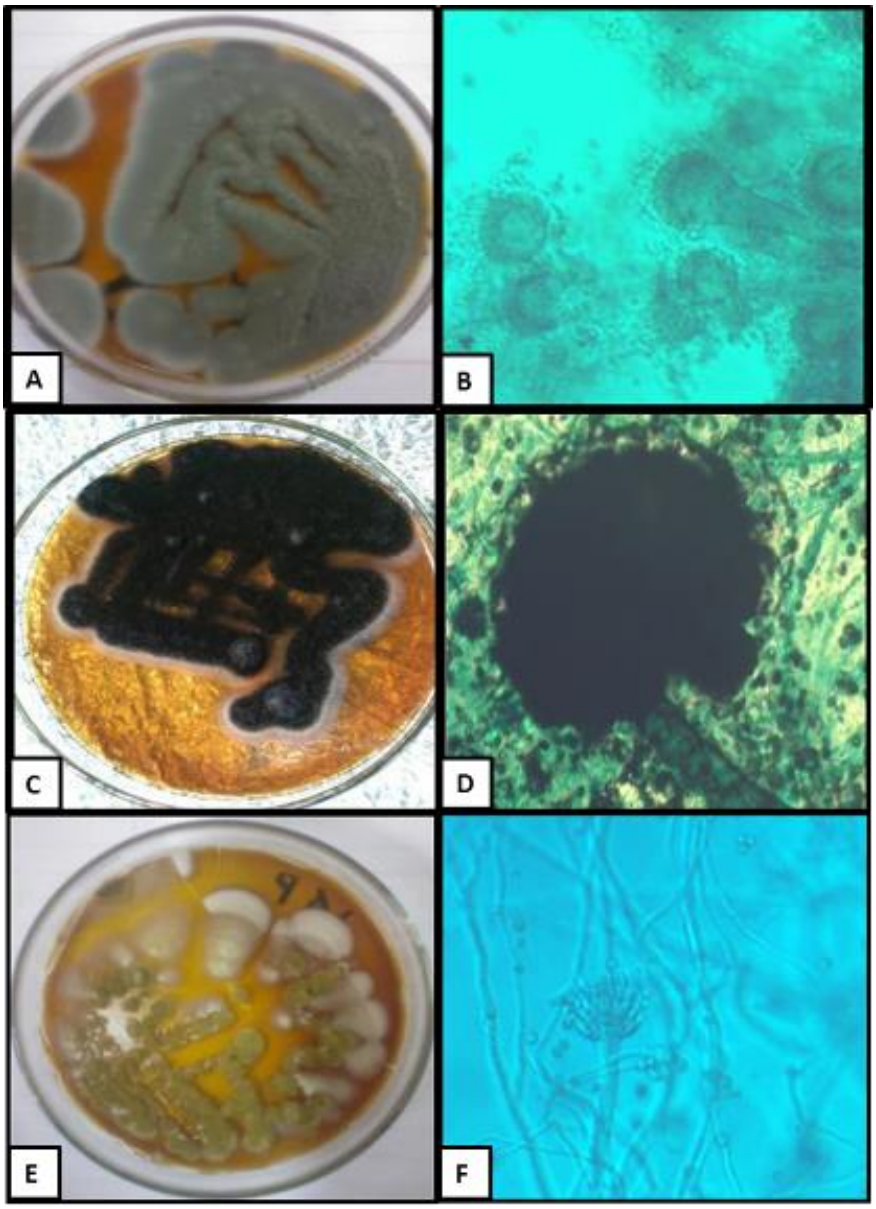

Fig.1. Macroscopical and microscopical appearance of isolated fungi.
A. fumigatus (A&B), *A. niger* (C&D), and *Penicillium spp.* (E&F).

Molecular detection of fungal isolates:

PCR amplification of ITS and ß-tubilin (benA) genes of Aspergillus spp.:

Figure 2 shows the results of PCR for DNA extracted from 8 randomly selected *Aspergillus* spp. isolates using ITS primer (A) and ß-tubulin gene specific primer (B). Results revealed that all the 8 isolates showed positive results with PCR test using specific primer that amplifies a 595-600 bp fragment of ITS region and a 492bp fragment of ß-tubulin gene that identified *Aspergillus* spp.

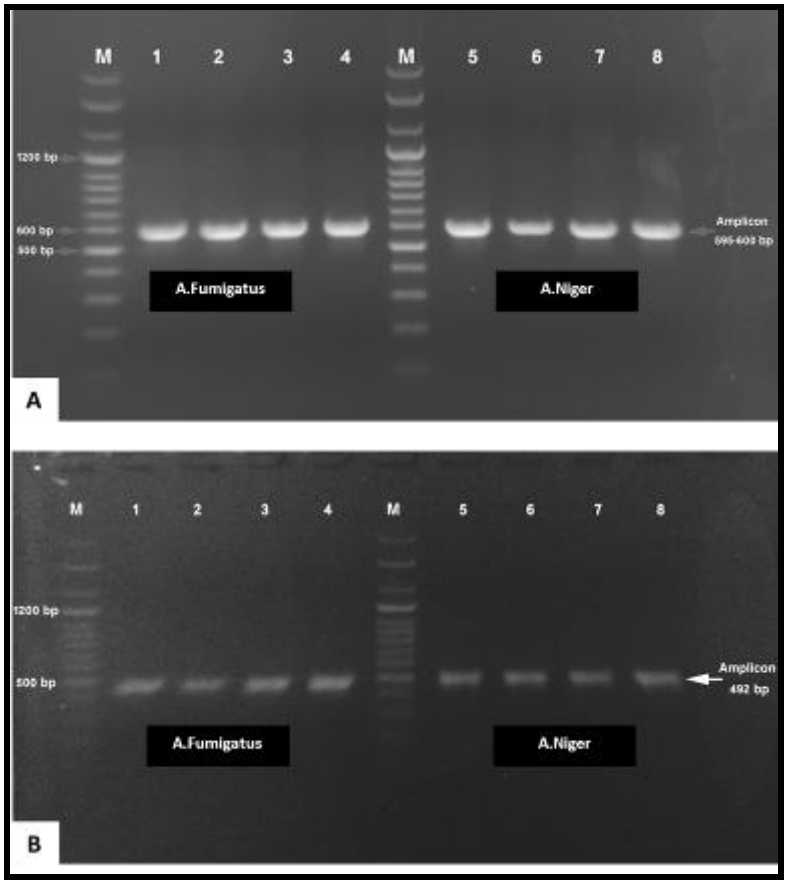

Fig. 2. Polymerase chain reaction amplification of (A) Internal Transcribed Spacer (ITS) region and (B) ß-tubilin gene (Ben A) of *Aspergillus spp.*, that were isolated from dead-in-shell embryos

Antifungal effect of different natural oil using agar dilution method:

Table 2 summarizes the results of the antifungal activity of the tested oils at concentrations of 1%, 0.5, 0.25, 0.1 and 0.05 against 20 randomly selected fungal isolates.

The cinnamon and thyme oils completely inhibited the growth of different fungal isolates at all concentrations 1%, 0.5, 0.25, 0.1 and 0.05 until 8 days of incubation. The clove oil completely inhibited the growth of different fungal isolates at all concentrations 1%, 0.5, 0.25, 0.1 and 0.05 until 8 days of incubation except for one *Penicillium spp.* isolate there was no antifungal activity for clove oil at a concentration of 0.05% as the fungal growth of this isolate was detected earlier after 4 days only of incubation.

In contrast, rosemary oil completely inhibited the growth of all tested fungal isolates at concentrations of 0.5 and 1%, while at the other concentration there were variable results of antifungal activity of the oil. At a concentration of 0.05%, there was antifungal effect with only one *A. niger* isolate. Increasing the oil concentration to 0.1% showed antifungal effect against only one *Penicillium spp.* isolate.

The 0.25% concentration was able to inhibit fungal growth of 4, 3, 1 and 1 isolate s of A. *fumigatus*, *A. niger*, *Penicillium* spp. and *Cladosporium* spp. respectively.

It was notable that the fungal growth in non-inhibited isolates usually occurred after 3 days of incubation for *Aspergillus* spp. and after 4 days for *Penicillium* spp. and *Cladosporium* spp.

TABLE 2. Antifungal effect of some essential oils against fungal isolates recovered from dead-in-shell embryos

Fungus	Sample ID	Oil dilution at which fugal growth was inhibited			
		Rosemary	Cinnamon	Clove	Thyme
A. fumigatus	35	0.50	0.05	0.05	0.05
	82	0.25	0.05	0.05	0.05
	102	0.25	0.05	0.05	0.05
	105	0.25	0.05	0.05	0.05
	110	0.25	0.05	0.05	0.05
A. niger	20	0.50	0.05	0.05	0.05
	17	0.25	0.05	0.05	0.05
	32	0.25	0.05	0.05	0.05
	37	0.25	0.05	0.05	0.05
	38	0.05	0.05	0.05	0.05
Penicillium spp.	16	0.50	0.05	0.05	0.05
	16x	0.50	0.05	0.05	0.05
	40	0.50	0.05	0.05	0.05
	61	0.25	0.05	0.10	0.05
	37	0.10	0.05	0.05	0.05
Cladosporium spp.	5	0.50	0.05	0.05	0.05
	6	0.50	0.05	0.05	0.05
	56	0.50	0.05	0.05	0.05
	74	0.50	0.05	0.05	0.05
	72	0.25	0.05	0.05	0.05

Discussion

The ability of fungi to invade the eggs and their efficient growth in embryonated eggs has been recently highlighted as a disease model for invasive fungi as compared to mouse model (Jacobsen *et al.*, 2010 & 2012). This study was designed to investigate the prevalence of fungal agents in dead-in-shell embryos. Moreover, the antifungal activity of extracted essential oils including, cinnamon, thyme, clove and rosemary was investigated.

Seventy nine fungal isolates were recovered in a rate of 66.9% of which *A. fumigatus*, *A. niger*, *Cladosporium* spp. and *Penicillium* spp. were represented in a prevalence of 28.0, 18.6, 11.9 and 8.5% respectively. Comparable results were previously reported where fungal isolates were represented about 41.7 % of the tested egg shell samples and *A. niger, A. fumigates* represented the majority of these isolates with 12.5% and 10%, respectively (Moustafa, 1995). Lower recovery rates of *A. fumigate, A. niger, A. flavus* and *Penicillium* spp. from eggs with early embryonic deaths (3.25, 4.75, 2.75, and 4.25%, respectively) were also reported (Enany *et al.*, 1989). The differences of recovery rates of fungi may be attributed to the isolation timing at early embryonic death. Additionally the sanitary condition of the hatcheries may play role in the recovery rate of contaminating fungi.

Other studies reported that the predominant fungi in dead-in-shell embryos were *A. niger* and *A. flavus* followed by *A. fumigates* and other fungal species (Osman, 1991). However, *Aspergillus* spp. remains the highest prevalent fungal species isolated from dead-in-shell embryos. This information further highlights the importance of the *Aspergillus* spp. as the major cause of egg hatchability problems in poultry farms.

Molecular techniques used for detection and characterization of *Aspergillus* spp. using the Internal Transcribed Spacer (ITS) region and β-tubulin gene of *Aspergillus* spp. have been also proven to be a useful target for discrimination of the most common pathogenic fungi. The two genes appeared to be a slowly evolving, conserved gene with a high degree of interspecies variability (Mirhendi *et al.*, 2007). All the 8 isolates showed positive results with PCR test with amplification of the 595-600bp fragment of ITS region and the 492bp fragment of ß-tubulin gene of *Aspergillus* spp., respectively. The results confirmed the conclusion stated by Kanbe *et al.* (2002) that ITS gene PCR method is rapid, simple and available as a tool for detection and identification of *Aspergillus* spp. pathogenic for poultry not only for *Aspergillus* spp. pathogenic for human with the same efficiency.

In this study we investigated the antifungal effect of 4 essential oils including cinnamon, thyme, clove, and rosemary on 20 fungal isolates of *A. fumigatus, A. niger, Cladosporium* spp., and *Penicillium* spp. (5 isolates each). A potent antifungal activity of cinnamon and thyme oils at the all tested concentrations as low as 0.05% up to 8 days of incubation. These results are in agreement with

those obtained by Cvek *et al.* (2010), who evaluated the effect of essential oils of cinnamon, lavender, rosemary, and sage at 1% concentration on spores of *Aspergillus* spp. and *Penicillium* spp. The study showed that cinnamon showed 100% inhibitory effect (100 %) with *Aspergillus ochraceus* and *Penicillium expansum*.

Clove oil was completely inhibited the growth of different fungal isolates at all concentrations and as low as 0.05% until 8 days of incubation with the exception of one *Penicillium* spp. isolate, where it was effective at 0.1% concentration which in accordance with the results which obtained by Passone *et al.* (2012) who reported 80.6% inhibited growth rate for *A. niger* and *A. carbonarius* by clove oil at 1500µl/liter (i.e. 0.15%) concentration in peanut meal extract agar and a fumigant activity at 3000 µl/liter (*i.e*. 0.3%).

In contrast, the rosemary oil showed variable degrees of antfungal activity. The rosemary oil completely inhibited the growth of different fungal isolates at higher concentrations (0.5 and 1%). At concentration of 0.25%, it was able to inhibit fungal growth of 4, 3, 1 and 1 isolate s of *A. fumigatus*, *A. niger*, *Penicillium* spp. and *Cladosporium* spp. respectively. These results are comparable to those reported by Fu *et al.* (2007) who tested the antimicrobial activity of the essential oils from rosemary and clove and their results showed the the minimum inhibitory concentrations of rosemary oil ranged from 0.125% to 1.0% (v/v) as compared to clove oil which ranged from 0.06% to 0.5% (v/v).

Analysis by gas chromatography showed that clove oil antifungal activity was attributed to its high content of eugenol (85.3 %) (Pinto *et al.,* 2009). Also thymol, is the major compound in thyme, and eugenol in cinnamon. Both thyme and eugenol are phenolic compounds (Suhr and Nielsen, 2003). These phenolic compounds were found to decrease conidiation, cause leakage of cytoplasm, loss of pigmentation and disrupted cell structure indicating fungal wall degeneration (Carmo *et al.,* 2008). However, the chemical compositions of rosemary oils revealed 22 components, of which 1, 8-Cineole represented 26.54% and α-Pinene was 20.14% (Jiang *et al.*,2011). This chemical composition indicated that there is no major antifungal component in rosemary oil as compared to other oils that may explain the variable results obtained by rosemary oil.

The differences observed between findings of different studies have been attributed to the amount of active compounds that reach the agar surface at a critical time (Gutierrez *et al.,* 2010). Other factors include the purity of extracted oils and the total amount of the active ingredients, the oil testing method, and the formulation of the essential oil (liquid or vapor) may play a role in determining the minimum inhibitory concentrations of oils (Tullio *et al.,* 2007).

In conclusion, *Aspergillus* spp. remains the highest prevalent fungal species isolated from dead-in-shell embryos indicating their importance as a major cause of egg hatchability problems. The essential oils such as cinnamon and thyme oil

could be used as antifungals with concentrations as low as 0.05%. Molecular diagnosis using PCR amplification of ITS and β-tubulin genes could be used as a fast tool for *Aspergillus* spp. diagnosis to overcome the limitations of morphological and microscopical identification techniques.

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الفطريات المصاحبة لنفوق الأجنة فى بيض الدجاج والرومي

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قسم البكتريا والفطريات والمناعة – كلية الطب البيطرى- جامعة بنى سويف- بنى سويف – مصر .

يمثل نفوق الأجنة فى البيض سبباً رئيساً من أسباب الخسائر فى مفرخات الدواجن ، هناك أبحاث محدودة عن دور الفطريات في مشاكل الفقس.
في هذه الدراسة تم عزل الفطريات المختلفة من الأجنة الميتة في البيض من السلالات المصرية المحلية. تم اختيار بعض المعزولات لتصنيفها جزيئياً. تم عزل تسعة وسبعون من المعزلات الفطرية بنسبة ٦٦,٩٪ منها أسبيرجلاس فوميجاتوس, أسبير جلاس نايجر ,كلادوسبوريم والبنيسيليم ممثلين ومثلت في نسبة انتشار ٢٨، ١٨,٦، ١١,٩ و٨,٥ بالمائة على التوالي. يبقى فطر الأسبيرجلاس هو أكثر الفطريات المعزولة من أجنة البيض النافقة.

لقد تم تصنيف المعزولات الفطرية بنجاح بإستخدام تفاعل البلمرة المتسلسل الذى يوظف بادئات محددة لمنطقة ITS وآخر لجين β-tubulin فى فطر الأسبيرجلاس.

في الختام أشارت هذه النتائج الى أهمية فطرالأسبيرجلاس في مشاكل الفقس فى الدواجن المصرية. وقد ثبت أن تفاعل البلمرة المتسلسل أداة سريعة وبسيطة لتحديد فطر الأسبيرجلاس . وعلاوة على ذلك يمكن استخدام الزيوت العطرية كمضادات للفطريات بكفاءة.