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MOLECULAR CHARACTERIZATION OF ASPERGILLUS AND YEAST IN BROILER CHICKENS

By

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ABSTRACT

A total of 220 chickens were collected from different commercial broiler farms (1- 30 days old chicks) at Dakhlia Governorate and inspected for mycotic infection. Organ samples were taken from 100 diseased chicken and 120 freshly dead ones (lung, air sac, crop, liver and brain from each chicken) after clinical and postmortem examination. All samples were cultured on specific media for fungi and examined macroscopically and microscopically together with the biochemical tests for identification of the fungi. The genotypic characters of fungi were done by using PCR. The results of fungi isolation revealed that 190 isolates (35.85%) out of 530 samples were positive for fungi; represented as 122 positive samples (36.97%) from diseased chickens and 68 positive samples (34 %) from freshly dead ones. Positive lung samples were 56 (29.47%) followed by 47 (24.74%) from liver and air sac samples while, 40 (34.78%) were from crop samples. Meanwhile isolates of *Aspergillus* (*A*.) spp. were higher than those of *Candida albicans* (*C. albicans*); *A. fumigates* was the frequently isolated spp. 87 (45.79%) followed by *A. flavus* 47 (24.74%); *A. niger* 38 (20%) and *C. albicans* 18 (9.47%), respectively. The molecular characterization of Aspergillus (A.) spp. and *C. albicans* was carried out by using PCR followed by sequencing of the PCR products. The identification of Aspergillus spp. and *C. albicans* by PCR was based on using 18S and 28S rDNA as target DNA. The sequences obtained for *A. flavus* isolate SR6 internal transcribed spacer 1, partial sequence of 28S ribosomal RNA gene obtained were more than 97% identical to the corresponding Gen Bank sequences. Finally, we concluded that *Aspergillus spp*. and *C. albicans* were the most isolated fungi and they were the most importantcauses of mould infection and candidiasis in broiler chicken farms.The characterization of DNA sequences was used as a diagnostic method to distinguish between different *Aspergillus* and different yeast.

INTRODUCTION

Mycotic infections are common in all kinds of poultry but are less prevalent as compared to bacterial and viral infections. Fungi are eukaryotic organisms, comprising both yeasts and molds. They cause significant economic losses to the poultry industry either due to their direct infectious nature or due to production of mycotoxins resulting in high morbidity and mortality rates, especially in young birds and cause stunted growth; diarrhea; and fatal encephalitis **(Singh *et al.,* 2012).** Aspergillosis is a necrotizing and granulomatous cavities disease of the lungs with haematogenous spread caused mainly by *Aspergillus fumigates*, the most pathogenic fungus affecting poultry **(Redig, 2005).** *A. fumigates* infection occurs more frequently in poultry, as the spores of this pathogen species are smaller than those of other Aspergillus spp. **(Dhama *et al.,* 2013b).** Candidiasis is a fungal disease caused by yeasts of the genus Candida having nearly 200 species, among them, six are most frequently isolated. While *C. albicans* is the most abundant and significant species*, C. tropicalis, C. glabrata, C.parapsilosis, C. krusei and C. lusitaniae* have also been implicated as causative agents of mycosis **(Tiwari *et al.,* 2011).** *C. albicans*is easily attached and penetrate into tissues. On the other hand, its cell wall glycoprotein composed mainly of mannan that has an endotoxin like activity **(Macdonald, 1984 and Dhama *et al.,* 2013b).** The identification of fungi by traditional microscopic, cultural and metabolic characteristics is still frequently used. Identification of the species level is very complex. Macro morphological identification is done based on conidial and mycelial color, colony diameter, colony reverse color, production of exudates and soluble pigments. Microscopic identification is mainly dependent on seriation, shape and size of vesicle, conidia and stipe morphology **(Chakranarayan and Pati, 2013)**.The methods used are PCR, fragment length polymorphism (FLP), restriction fragment length polymorphism (RFLP), DNA probe hyperdezation and DNA sequences **(Khaphagy *et al.,* 2012 and Zhao and Perlin, 2013).** The present work was planned to study the mycotic infection in broiler chicken to achieve the following steps were done:

1.Study of the incidence of the most common fungal infection in broiler chicken.

2.Phenotypic characterization of Aspergillus species and Yeasts isolated from internal organs of chicken.

3.Genotypic characterization of Aspergillus species and Yeasts isolated from internal organs of chicken.

MATERIAL AND METHODS

Samples and sampling:

A total of 220 chickens were collected from different private broiler farms (1 - 30 day old) at Dakahlia Governorate, they were inspected for mycotic infection. Samples were taken from organs of 120 slaughtered diseased chicken and 100 freshly dead ones (lung, air sac, crop, liver and brain from each chicken) after clinical and postmortem examination. Each examined organ was taken in a sterile plastic bag, and transferred to the laboratory for mycological examination (Table 1).

Table (1): Samples from diseased chicken and freshly dead ones collected from broiler chickens.

Chicken cases	No. of chicken	No. of Samples				
		Lung	Air sac	Crop	Liver	Total
Diseased chicken.	120	85	90	75	80	330
Freshly Dead chicken.	100	50	50	40	60	200
Total	220	135	140	115	140	530

Isolation and phenotypic identification of Aspergillus species:

Samples were streaked on Sabouraud's dextrose agar (SDA) plates and incubated for 10 days at 25° c with continuous observation of plates for any fungal growth. Colonies were picked up and kept in Sabouraud's dextrose agar slopes and then stored in refrigerator for further identification **(David *et al.*, 2007).**

Isolation and phenotypic identification of yeast:

Samples were streaked on Sabouraud's dextrose agar (SDA) plates and incubated for 48h at 30°C then at 37◦C for further 48 hrs *(***Kotb, *et al.* 2008 and Sheimaa, *et al.*, 2011)**. The yeast colonies were picked up and kept in Sabouraud's dextrose agar slopes then incubated at 30°C for 48hrs. The slopes were stored in refrigerator for further identification **(Sivakumar *et al.*, 2008).**

Identification of suspected Aspergillus and yeast isolates:

It was carried out by the macroscopic and microscopic examination for specific characteristics of the colonies according to **Markey *et al.* (2013).**

Extraction of DNA from Aspergillus spp:

According to QIAamp DNeasy Plant Mini kit instructions.

Preparation of conventional PCR Master Mix:

According to Emerald Amp GT PCR mastermix (Takara) Code No. RR310A kit.

Extraction of DNA from *Candida albicans*:

According to QIAamp DNA mini kit instructions.

Preparation of PCR Master Mix:

According to Emerald Amp GT PCR mastermix (Takara) Code No. RR310A kit.

Agarose gel electrophoreses:

It was carried out as stated by **(Sambrook *et al.*, 1989)**.

Method of sequencing:

Isolates were purified with Gene jet PCR purification kit, Ferments (Cat. No. K No.K1080, USA.) and sequenced by Chromogen Company, Germany **(Sanger *et al.*, 1977)**.

RESULTS

Table (2): Total number and Percentage of positive samples for fungal isolation from broiler chickens.

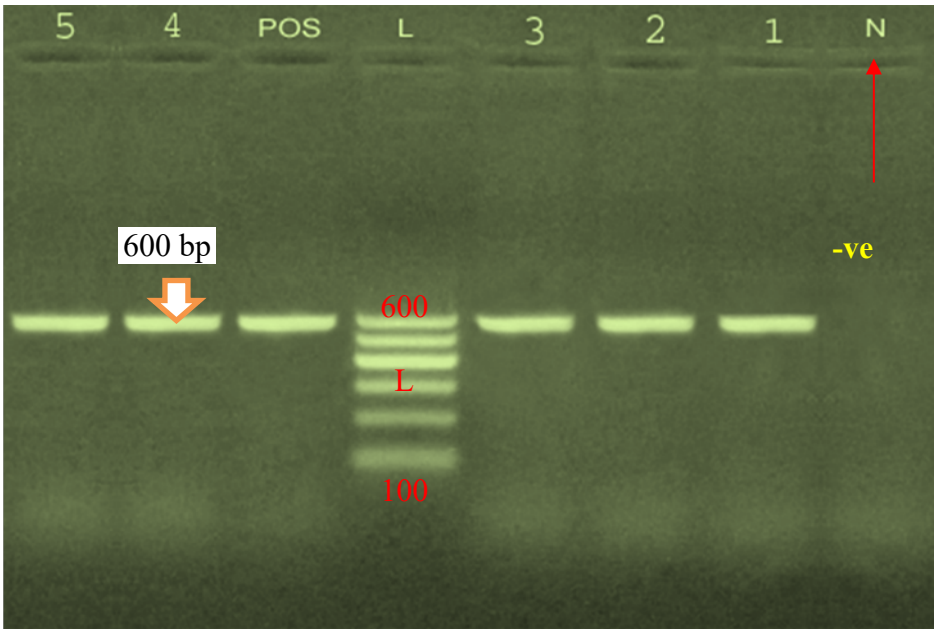
Chicken case	Number of sample	Number of positive samples				Total No. of Positive %
		Lung %	Air sacs %	Crop %	Liver %	
Diseased Chicken	330	34/85 40%	32/90 35.56%	24/75 32%	32/80 40%	122 36.97%
Freshly dead Chicken	200	22/50 44%	15/50 30%	16/40 40%	15/60 25%	68 34%
Total	530	56 29.47%	47 24.74%	40 22.11%	47 24.74%	190 35.85%

Table (3): Number and percentage of fungi isolated from different organs of broiler chickens.

Fungus	Lung %	Air Sac %	Crop %	Liver %	Total %
A. fumigatus	30 34.48%	25 28.74%	12 13.79%	20 22.99%	87 45.79%
A. flavus	15 28.85%	12 27.27%	8 21.62%	12 28.57%	47 24.74%
A. niger	9 18.42%	9 23.68%	13 39.47%	7 18.42%	38 20%
C. albicans	2 3.85%	1 2.27%	7 18.92%	8 19.05%	18 9.47%
Total	56 29.47%	47 24.74%	40 22.11%	47 24.74%	190 35.85%

Table (4): Biochemical and physiological reaction of *C. albicans*

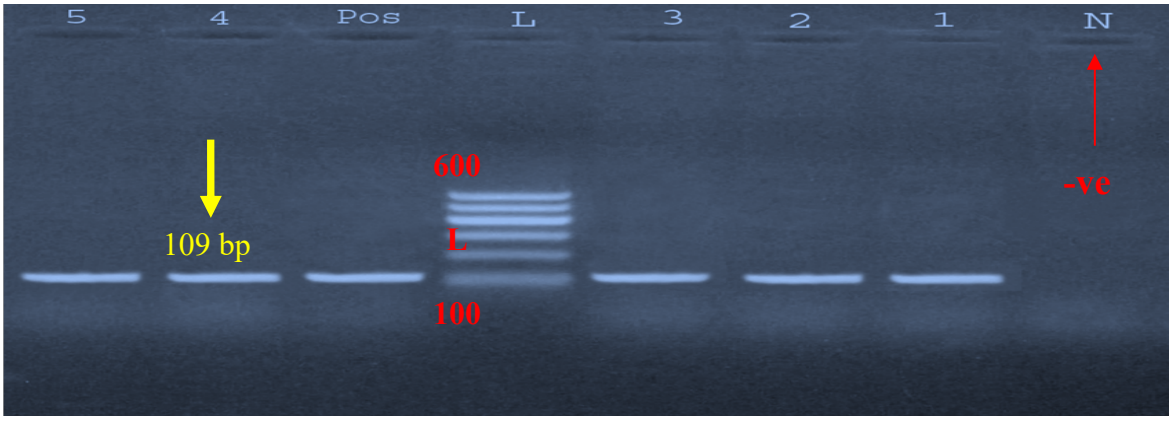
Physiological Tests	*C. albicans*
Germ Tube Test	+
Sugar fermentation: Glucose	+
Galactose	v
Sucrose	- (s)
Maltose	+
Lactose	_
D-Mannitol	+
Soluble Starch	+
Nitrate reduction	_
Urease hydrolysis	_



Lane L.: 100-600bpDNA ladder**Lane N:** negative control

Lane POS: positive control**Lane 1-5:** positive Aspergillus

Fig (1): PCR using fungus-specific universal primer pairs (ITS1 and ITS4) was used for identification and genotypic characterization of Aspergillus species. All tested Aspergillus isolates, provided a single compatible electrophoretic band about 600 bp.



Lane L.: 100-600bpDNA ladderLane N: negative control

Lane POS: positive control at 109bpLane1-5; Positive *C. albicanis*

Fig (2):PCR using fungus-specific universal primer pairs (ITS and RPS) was used for identification and genotypic characters of *C. albicans*. All tested *C. albicans* isolates, providing a single PCR product of about 109 bp.

Nucleotide Acid sequence forITS-1 region of A. flavus:

CGGGTGTACGGTTCTAGCGAGCCCACCTCACCCCCCACCCGTGTTTACTGTACCTT
AGTTGCTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCC
CGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTCGTCAAGTCTGAGTTCAT
TGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGAT
GAAGAACGCAGCGAAATGCGATAACTCGTGTGAATTGCAGAATTCCGTGAATCA
TCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCC
GAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTC
CGGGGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGT
ATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAATC
AATCTTTTTCCAGGTTCACCTCGGATCAGGTCGGGATACCCGCTGAACTTAAGCA
TATCAATAAGGCGGAGG

Amino acid sequence for ITS-1region of A. flavus:

RVYGSSEPTSPPTRVYCTLVASAGPPFMAAGGSQPRARARRRHHELCLISSSLSSLYR
NQLKLSTMDLLVPASMKNAAKCDNSCELQNSVNHRVFERTLRPLVFRGACLSERHC
CPSSTACVLGRRPLSGGDGPQRQRRHRVRSSSVWGFVTRSVGPAGACRTQINLFPGSP
RIRSGYPLNLSISIRRR

Nucleotide Acid sequence for ITS-1region of *C. albicanis:*

GATGAACCCCATGTGCTACAAAGACCAAACTCGGGCCGTTTTGAAGCTACAATCA
TGTATAGTATTGGGTGTGAATTAGGCATGAATCGGATCAGAATTGGTTTAGCTAT
TGAAGAAAACGTTTTCTCCGTGGAAATGTGTAATTATCTCCGCCAAGGCTGTCAC
AGTCAGTTTCGATGCTATAAAGACCCAACTAGTGCCAATTCATATCCATATGATG
TAAGTACTATGACTGTAAGAGCTGTTAGAAACAAGGTTCAACTGCTTTCTGTAGA
ACAAAAAAGGCCGTTTTTGCCATATTTAAGGAATTCGCGGTGTTGTCCGTTGAAGACTGC
GCGATGTAAAATAACGCTACAAAAATCAAACTCGTGCCGATTTATACCTTTTTCTTATGA
GTGCTCACCATGCAAGAACTGTTTGAAACGAAATACAACTGCTATCTGTGGAACAAAAA
AGGCCGTTTTGGCCATAGTTAAGGGAGCCGCAGCTATGTCTGATCACAACTACGCGACCA
AATTCAACGCTACAAAAATCAAACTAGTGCCGATTTATACCTTTGGATTATATGTTCTA
TCCCTGCAAGAACTGTTAGAAACGAAATTTAACTGCTTTCTGTGGAACAAAAAAGGC
CGTTTTGTCCATAGTTTAGAGGGAAAAATTATGTATATTGTTGACAGAAGATCGAATTTG
AATGAGTTAATGACAAGGCTAGTATCGATTTGGAACCACAAAATGTGTGTGTCAAAGCCG
TGGGATACTGTTAGAAAAGAGATACAACTGCATACCGTGGGACAAAAAAGGCCG,

Amino acid sequence for ITS-1region of *C. albicanis:*

DEPHVLQRPNSGRFEATIMYSIGCELGMNRIRIGLAIEENVFSVEMCNYLRQGCHSQF
RCYKDPTSANSYPYDVSTMTVRAVRNKVQLLSVEQKRPFLPYLRNSRCCPLKTARC
KITLQKSNSCRFIPFSYECSPCKNCLKRNTTAICGTKKAVLAIVKGAAAMSDHNYATK
FNATKIKLVPIYTFGLYVLSLQELLETKFNCFLWNKKGRFVHSLEGKIMYIVDRRSNL
NELMTRLVSIWNHKMCVSKPWDTVRKEIQLHTVGQKRP.

DISCUSSION

Mycotic infection of chicken is one of the most serious problems that affect chicken causing high economic losses due not only to the high morbidity and mortality in young chicken but also they are the leading cause of immunosuppression in birds **(Arne *et al*., 2011)**. On the other hand, an increase in the incidence of the mycotic diseases can be expected due to the wide use of antibiotic preparations in the treatment of many diseases as well as the extensive use of antibiotic as feed additives, which enhance mycotic complications. Therefore, this study was planned for mycological examination in broiler chicken farms (**Musa *et al*., 2014).**

The results of fungal isolation revealed that 190 positive samples (35.85%) out of 530 samples were positive for fungal isolation; represented as 122 positive samples (36.97%) from diseased chickens and 68 positive samples (34 %) from freshly dead ones. Moreover, the highest positive samples in diseased and freshly dead chickens were in lung (29.47%) and air sac and liver samples (24.74%) respectively (Tables 2 and 3) these results came in accordance with those obtained by **(Garcia *et al.,* 2003 and Sajid *et al.,* (2006).** The results represented in (Table 3) revealed that, a total number of 190 fungal isolates were isolated from 530 samples, where 56 (29.47%) were isolated from lung samples followed by 47 (24.74%) from air sac and liver samples while, 40 (21.05%) from crop samples. Meanwhile *A. fumigatus* was the most isolated species 87 (45.79%) followed by *A. flavus* 47 (24.74%); *A. niger* 38 (20%) and *C. albicans* 18 (9.47%) these results agreed with **Tartor, (2010); Lorin, (2013) and Salem and Ali**, **2014).** *Aspergellus* species were isolated 172 (90.53%) mostly from lung samples (31.40%), followed by (26.74%), (22.67%) and (19.19%) from air sacs, liver and crops samples respectively. Moreover, Candida species isolated were 18 (9.47%) mostly from liver samples (19.05%) followed by (18, 92%), (3.85%) and (2.27 %%) from Crop, lung and air sac samples respectively (Table 3). These results agreed with **Steinlage *et al.,* (2003) and Musa *et al*., (2014).** The molecular characterization of

Aspergillus spp. and *C. albicans* was carried out by using PCR and sequencing of the PCR products. The identification of Aspergillus spp. and *C. albicans* depending on PCR was based on using 18 S or 28 S rDNA as target DNA. However, the sequences in these regions are conserved across a wide range of fungi. The ITS region contains variable elements that allow sequence-based identification of Aspergillus species and *C. albicans*; therefore, the region offers a possible template for identification of different Aspergillus spp. and *C. albicans* either by using primers designed from this region for the different spp. or sequencing of the amplified region by using primers for amplification of the ITS region **(Lim and Lee, 2000 and Makimura, 2001).** Regarding *C. albicans*, the primers used for identification by PCR were designed for amplification of the ITS region. The examined samples were identified as *C. albicans* by the traditional methods and all samples were successfully amplified a product of 109 bp and give + ve with the PCR examination which confirm the results of the traditional methods **(Tarini *et al.,* 2010)**. The sequences obtained for *A. flavus* isolate SR6 internal transcribed spacer 1, partial sequence and 28S ribosomal RNA gene showed more than 97% identity to the corresponding Gen Bank sequences which agrees with **Ehrlich *et al.* (2007)** who recorded that Aflatoxin-producing Aspergillus species which were isolated from soil samples 2%**. Al-Harthy (2014)** was recorded the sequence of *A. flavus* genomic DNA containing ITS1, 5.8S rRNA gene and ITS2, strain TUHT120 isolated from some feedstuffs from the Western region of Saudi Arabia bases 1 to 637 and has accession number LN482516.1 and **Lai, (2015)** who isolated *A. flavus* KP689246.1, identify level is 98%. *Candida albicans* were subjected for sequencing; and the obtained results came in accordance with those recorded by **Lim and Lee (2000) and Tarini *et al.,* (2010).** The sequences obtained for *C. albicans* clone 39.1-13.7 Ca3 fingerprinting probe hyper variable band fragment, were more than 95% identical to the corresponding GenBank sequences and agree with **Iwaguchi *et al.,* (1992) and Tait *et al.,* (1997)** who have accession number emb AL033396.1 and also **Chibana *et al*., (2005)** who have accession number AP006852.1. Finally, we conclude that *Aspergillus* species, mainly *A. fumigatus; A. flavus; A. niger* and *C. albicans* were the most isolated fungi from broiler chickens and they were the most important causes of mould infection and candidiasis in broiler chicken farms. In addition, we could conclude the importance of ITS-1 region sequence in comparison between the different *Aspergillus* species and different yeast species. The characterization of DNA sequences was used as a diagnostic method to distinguish between different *Aspergillus* and different yeast species.

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التوصيف الجزيئي للاسبرجيليس والخمائر في بداري التسمين

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***قسم الدواجن – معهد بحوث الصحة الحيوانية – معمل المنصورة الفرعي**

****معهد البحوث التناسلية – الهرم – الجيزة – القاهرة**

الملخص العربي

عدوى الفطريات من أهم الأمراض التي تؤثر في صناعة الدواجن والتي تسبب خسائر اقتصادية كبيرة ليس فقط نتيجة نفوق الدواجن والفقد في الإنتاج والإعدامات في المجازر ولكنها عامل مساعد للإصابة بكثير من الأمراض الأخرى .

ولذلك فقد تمت هذه الدراسة على 220 طائر من عمر (1-30 يوم) تم تجميعهم من مزارع بدارى التسمين (من عمر 1-30 يوم) من محافظة الدقهلية.

تم اخذ 100عينه من طيور مريضه و120عينه من طيور حديثه الوفاه من (الرئه والأكياس الهوائية والحويصلة والكبد و المخ). وأظهرت نتائج الفحص أن 190 (35,85%) معزولة من 530 عينه موجبه للفطريات وكانت تمثل 122 عينه موجبه من الطيور (36.97%) من الطيور المريضة و68عينه (34%) من الطيور حديثه الوفاة .

وتم عزل 190 معزولة فطريات من 530 عينه وكانت كالتالي 56 (29,47%) عزلت من الرئة تلاها 47 عينه (24,74%) من الكبد والأكياس الهوائية بينما 40عينه مثلت (34,78%) من الحويصلة .

وأظهرت النتائج أن الاسبرجيليس كانت أكثر ظهورا من الكانيدا البيكان, والاسبرجيليس فيوماجس كانت أكثر معزولة مثلت 87 (45,79%) تلتها اسبرجيليس فيلافس 47 (27,74%) واسبرجيليس نايجر 38 (20%) وكانديدا البيكان 18 (9,47%) .ولقد كانت الصفات المورفولوجيه للفطريات والخمائر متطابقة مع الدراسات السابقه . ولقد تم تطبيق تفاعل البلمره المتسلسل للتعرف علي الصفات الجينيه لفطريات الاسبرجيليس فيوماجس فلافس والاسبرجيليس نيجر وتم الحصول علي ناتج من عمليات البلمره المتسلسل وزنه الجزيئي 600 من كل العينات التي تم فحصها أما بالنسبة لخمائر الكانديدا البيكان فكان الوزن الجزيئي 109.

وأخيرا يتضح من نتائج الدراسة أن فطريات الاسبرجيليس وبخاصة الاسبرجيليس فيوماجس والاسبرجيليس فلافس والاسبرجيليس نيجر وخمائر الكانديدا البيكان هما السبب الرئيسي في حالات الإصابة الفطرية في مزارع تسمين الدواجن وان تطبيق تفاعل البلمره المتسلسل أفضل الطرق لتشخيصها من حيث يالوقت والجهد والتكاليف .