Morphotypic and Molecular Detection of Fungal Agents from Local Egyptian Breeder Chickens, Hatching Eggs and Hatched Chicks

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ABSTRACT

In this study the fungal species were investigatd in local Egyptian breeds, their hatching eggs and hatched chicks. A total of 208 samples were collected from different breeder chickens, feed, water, incubators, dead-in-shell embryos, and hatched chicks of different local Egyptian chicken breeds. The samples were subjected to fungal isolation, morphotypic and molecular detection of Aspergillus spp. ITS and and β-tubulin genes. Resulst showed that, the highest prevalence of fungal isolates was reported in tracheal swabs collected from breeder chickens (42.8-57.1%) in all breed except Sainaa and Dahaby breeds that showed 0% and 14.2% isolation rate from tracheal swabs and 50% and 85.7% from cloacal swabs, respectively. In feed and water samples, 27.3% were positive for fungal isolation. Swab samples collected from incubators that incubating the eggs of 2 breeds (Dokki and Sainaa) yielded only Zygomycetes. The dead-in-shell and hatched chick's samples were collected from 4 breeds (Dokki, Sainaa, Gemeza and Dahaby) showed higher isolation rates (80-100%) 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. The most prevalent fungi in tracheal swabs were A. Niger (14.3%) and Zygomycetes (11.9%), while A. Terreus and A. Flavus showed similar rate of 4.8%. A. Terreus (17.5%) and Zygomycetes (12.5%) were the predominantly isolated fungi from cloacal swabs followed by both A. Flavus and A. Niger (10% each). The ITS and β-tubulin PCR was positive for the selected most predominant fungal species (A. Flavus, A. Niger, and A. Terreus). To summarize, the current study further confirmed the predominance of filamentous fungi (Aspergillus spp) in poultry production premises. The molecular detection using PCR of ITS and β-tubulin is an easy time-saving method for detection of Aspergillus species, however, further techniques are needed to discriminate between Aspergillus strains.

Keywords: Aspergillus; Layer Chicken; Hatchery; ITS; β-Tubulin

INTRODUCTION

Fungi are characterized by ubiquitous occurrence, thus animals and human are constantly exposed to the fungal spores. Several species have been described as animal and human pathogens with variable clinical presentations. Recent studies indicated that fungal infection can cause hatchability problems, contaminate table eggs and pose a potential zoonosis for human through airborne spores [1,2]. The suitability of chicken embryonated eggs for growth of pathogenic fungi has made them an infection model to study Aspergillus fumigatus virulence [3,4].

These data further confirmed the capability of these fungi to invade the eggs and its potential to be a primary cause of hatchability problems. Cafarchia and co-workers, reported the ability of Aspergillus species to penetrate via egg shell during incubation and subsequently to recently hatched chick [5]. Studies reported the contamination of the eggs with Aspergillus species with relatively high rates of 14% [6] and 66% [7]. Considering the high humidity and optimal temperatures especially when contaminated eggs are hatched [2], unclean incubators were proposed as the primary cause for the development of avian aspergillosis outbreaks in chicks.

Though morphological and microscopical identification of Asper gillus spp. has been well established to characterize the different fungal species. The main limitations of classical morphotypic diagnosis of fungi are laborious work and the need for experience for correct differentiation between fungi. Hence, molecular techniques including PCR amplification of targets and fragment length analysis, DNA probe hybridization or sequence analysis are being used to minimize the time and efforts consumed in the classical mycological procedures.

The current study was designed to investigate the prevalence of fungal isolates in local Egyptian breeder chickens, their hatching eggs and hatched chicks. Moreover, the PCR detection of both Internal Transcribed Spacer (ITS) and β-tubulin genes was em ployed for in comparison to the classical mycological techniques for fungal isolates detection.

MATERIAL & METHODS

Samples and samples processing

A total of 208 samples were collected from different breeder chickens, feed, water, incubators, dead-in-shell embryos, and hatched chicks of different local Egyptian chicken breeds. The samples were obtained from an automatic hatchery (El-Azab Integrative Poultry Project, Fayoum Governorate, Egypt). Detailed description of samples is summarized in figure 1. All samples were subjected to mycological examination at the laboratory of Bacteriology, Mycology and Immunology Department, Faculty of Veterinary Medicine, Beni-Suef University.

Isolation and morphological characterization of fungal agents

Samples were inoculated into Sabouraud's dextrose broth and incubated for 3-5 days at 27°C. A loop full from selective broth was plated onto Sabouraud's dextrose agar which previously prepared with adding chloramphenicol 50 mg/liter dissolved in 10ml ethyl alcohol. Incubated for 3-5 days at 27°C. Colony morphology of fungal isolates was conducted according to [8]. For microscopical examination fungal isolates were stained with lactophenol methylene blue stain [9].

PCR of ITS and β-tubulin genes of fungal isolates

The DNA was extracted using Gene-JET Genomic DNA Purification Mini Kit (Thermo Scientific, Lithuania) according to the manufacturer instructions and as previously described [7]. Oligonucleotide primers targeting the ITS and β-tubulin genes of the genus Aspergillus were used [10, 11].

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 of both

reactions is summarized in Table 1. 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.

RESULTS

Fungal species recovery rate from breeder layers, feed and water, dead-in-shell embryos, and hatched chicks

The results of fungal isolation from breeder layers, feed and water are shown in figure 1. The total rate of isolation from tracheal and cloacal swabs was 42.9 and 42.5%, respectively. Gemeza and Sasso breeds showed the highest isolation rate from tracheal swabs (57.1%) while Dahaby and Gemeza breeds showed the highest incidence of fungal isolation (85.7% and 50%, respectively). Both feed and water samples were positive only in Sainaa and Gemeza breed's houses, however, only water samples collected from Sasso houses and feed samples collected from Dahaby breed houses were positive for fungal isolation.

Swab samples collected from incubators were only available for those incubating eggs of Dokki and Sainaa breeds. The results of these samples showed 50% fungal isolation rates. Meanwhile dead-in-shell and hatched chick's samples were collected from 4 breeds (Dokki, Sainaa, Gemeza and Dahaby). Higher isolation rates of fungi were observed from dead-in-shell eggs (80%) of Dokki and Sainaa breeds. Also, lungs and livers of these two breed also showed 80-100% isolation rates. In contrast, Dahaby and Gemeza breeds the isolation rate from both dead-in-shell eggs and hatched chick's organ samples were low (0-20% and 0-40%, respectively) (figure 1).

Fungal species recovered from breeder chickens, their feed and water, dead-in-shell eggs and hatched chicks in relations to species and breed

Fungal species recovered from breeder layer's tracheal and cloacal swabs are shown in figure 2. The most prevalent fungi in tracheal swabs were A. niger (14.3%) and Zygomycetes (11.9%), while A. terreus and A. flavus showed similar rate of 4.8%. A. terreus (17.5%) and Zygomycetes (12.5%) were the predominantly isolated fungi from cloacal swabs followed by both A. flavus and A. niger (10% each). Cladosporium spp. was isolated only from cloacal swabs with a very low rate (2.5%). A. terreus was isolated from 3 out of 11 feed samples tested (27.3%) and both A. niger and Zygomycetes were isolated in only 1 samples each (9.1%). A. niger and Zygomycetes were also the predominant fungal isolates from water samples. A. flavus was not isolated from either feed or water samples.

None of the veterinary important fungi was isolated from incubators but only Zygomycetes. Out of 60 dead-in-shell eggs samples tested, 10 were positive for A. terreus, 10 were positive for A. flavus (16.7% each) and 4 were positive for A. niger (6.7%). 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 the total isolation rates were 18.3% for both A. flavus and A. niger, 13.5% for A. terreus, then a low rate of 0.96% and 1.9% for Cladosporium spp. and Zygomycetes, respectively.

Growth and morphological criteria of the isolated fungi

The isolated A. flavus showed slow growth of 3 to 4 cm colonies. The colonies consisted of a close-textured basal mycelium which is flat or rapidly furrowed or wrinkled. Conidial heads are abundant and intense yellow to yellow-green color. The reverse is colorless to pinkish drab or darker. Microscopical examination revealed thick-walled un-pigmented, coarsely roughened conidiophores, long (up to 1 mm in length or more) globose or sub-globose vesicles producing phialides over almost the entire area in two rows (figure 3 A-C).

Aspergillus terreus colonies appeared as rapidly growing powdery colonies with a characteristic cinnamon-brown color on the surface. The reverse color was yellow to beige-brown. Microscopically, A. terreus revealed hyaline and septate hyphae, conidiophores are smooth walled and hyaline, biseriate phialides, extending from the upper portion of the vesicle, conidia form in long chains are round, smooth walled with broom like conidiophores (figure 3 D-F).

Typical Aspergillus niger colonies were produced within 2 to 4 days. Growth began initially as a white colony that soon developed a black, dotted surface as conidia were produced and with age the colony became jet black and powdery, whereas the reverse color was olive-yellow. Microscopically, A. niger exhibited septate hyphae, long conidiophores supporting spherical vesicles that gave rise to large metulae and smaller phialides from which long chains of brown rough-walled conidia were produced (figure 3 G-I). Other fungi of low prevalence included Cladosporium and Zygomycetes species showed their typical macroscopic and microscopic and microscopic appearances (Data not shown).

PCR amplification of the ITS and β-tubilin genes of the isolated Aspergillus spp

The PCR amplification of 7 samples from A. flavus isolates from breeder chicken breeds (4 isolates) and their chicks (3 isolates) using ITS primer. Three isolates from breeder chickens were positive while 2 isolates from chicks were positive. The PCR product size is 595-600bp characteristic for the ITS region of Aspergillus spp. Out of 7 samples from A. terreus isolates from breeder chicken breeds (4 isolates) and their chicks (3 isolates) using ITS primer. Only 2 from each were positive. The PCR product size is 600bp. The 4 selected isolates (2 from breeder chicken breeds and 2 from their chicks) were positive for ITS primer (595-600bp) (figure 4).

DISCUSSION

The aim of this study was to investigate the prevalence of fungal agents in local Egyptian breeds, their hatching eggs and hatched chicks. In breeder chicken farms, the highest prevalence of fungal isolates was reported in tracheal swabs collected from breeder chickens and ranged between 42.8-57.1% in all breed except Sainaa and Dahaby breeds that showed 0% and 14.2% isolation rate from tracheal swabs and 50% and 85.7% from cloacal swabs, respectively. The higher rate of isolation from the respiratory tract may be explained by the small diameter of the Aspergillus conidia ranges (2-3µm) allowing them pass through physical barriers of the respiratory tract [5, 12].

Though few feed and water samples were tested, out of 22 feed and water samples collected only 27.3% were positive for fungal isolation. The 3 samples were restricted to Sainaa, Gemeza and Dahaby breeds. The three breeds showed the highest cloacal isolation rate indicating potential feed contamination with feces [13, 14]. Recent studies reported the contamination of the eggs with Aspergillus species with relatively high rates of 14% [6] and even up to 66% [7]. The role of incubators is essential in the development of avian aspergillosis outbreaks in baby chicks. The high humidity and optimal temperatures can promote the development of mold especially when contaminated eggs are hatched [2]. A. terreus was less frequently isolated from poultry from feed stuff [15, 16]. However, the ubiquatos nature of A. terreus in the environment and belongs to the section Terrei [17] may explain the isolation of A. terreus with high prevalence rate in both breeder chickens and their hatching eggs and chicks.

Swab samples collected from incubators that incubating the eggs of Dokki and Sainaa breeds showed 50% fungal isolation rate (2 out of 4 samples) (table 1). The positive samples yielded the isolation of only Zygomycetes. The low number of samples and the difficulty to collect samples on timely manner make it difficult to judge the hygienic conditions of the incubators or to track the potential growth of fungi in the incubated eggs. The dead-in-shell and hatched chick's samples were collected from 4 breeds (Dokki, Sainaa, Gemeza and Dahaby). 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 breeds dead-in-shell eggs and hatched chick's organ samples showed lower.

Previos studies indicated the use of embryonated eggs for Aspergillus species growth has been reported [3,4]. Also the filmnrous fungi were reported to penetrate the egg shell during storage [1]. Thus high fungal isolation rates from dead-in-shell is associated with high isolation rate in hatched chick further confirm the important role played by the hatchery environment in the spread of avian aspergillosis [1,2,18].

A. fumigatus and A. flavus are the most frequently isolated from animal, avian and human infections and both are the etiological agent of Aspergillosis [20,21]. Generally, the species of the genus Aspergillus remained the most isolated fungi in breeder chickens and their hatched chick's samples collected. The Aspergillus species are known to favorably grow in poultry houses producing large amounts of spores [22,23,24]. Humidity and temperature of the poultry farms promote the efficient asexual growth of fungi producing enormous amounts of airborne conidia that is easily inhaled by birds [25,26].

Though the morphological and microscopical identification of Aspergillus spp. has been well established and enabled us to characterize the different isolated Aspergilli, but they are of low benefit for identification on the level of the strains to track the potential source of isolated fungi. Thus various molecular approaches have been used for the identification of Aspergillus spp. [27,28,29]. The β-tubulin gene is involved in both vegetative growth and asexual sporulation of the Aspergillus spp. [30,31]. Also, the ITS gene is widely used for molecular detection of fungal isolates. Therefore, PCR of both genes was applied to identify selected isolates of the most predominant 3 fungal species A. flavus, A. niger, and A. terreus. Selected samples for PCR were Aspergillus flavus (4 isolates) and Aspergillus terreus (4 isolates), and Aspergillus niger (2 isolates) distributed equally between breeder chickens and their eggs &hatched chicks. As previously reported, the the ITS and β-tubulin amplicons produced were 600bp and 492bp, respectively [10,11].

In conclusion, the current study further confirmed that the filamentous fungi (Aspergillus spp) is the most isolated fungi in breeder chickens and their incubatedeggs and hatched chick. The molecular detection using PCR of ITS β-tubulin is an easy time-saving method for detection of Aspergillus species, however, further techniques are needed to discriminate between Aspergillus strains. Surviellence of fungal contamination in poultry production premesis is required to monitor the hygiene condition in oultry hatcheries to avoid the spread to newly hatching chicks.

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Table 1: Thermal PCR profile for ITS and ß-tubilin regions.

Gene	Initial denaturation	35 cycles			Final extension	Amplicon size
		Denaturation	Annealing	Extension		
ITS	94°C for 5m	94°C for 30s	56°C for 45s	72°C for 1m	72°C for 7m	595-600bp
β-tubulin	94°C for 5m	94°C for 30s	56°C for 45s	72°C for 1m	72°C for 7m	492bp

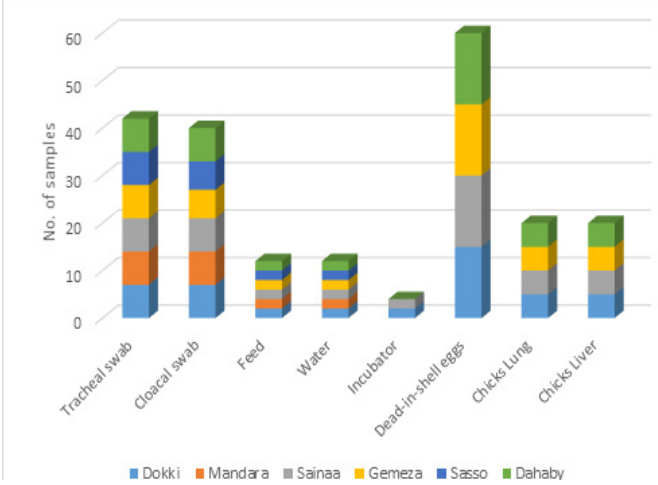


Figure 1. Type of samples collected from different chicken breeds farms, incubators, and their hatched chicks.

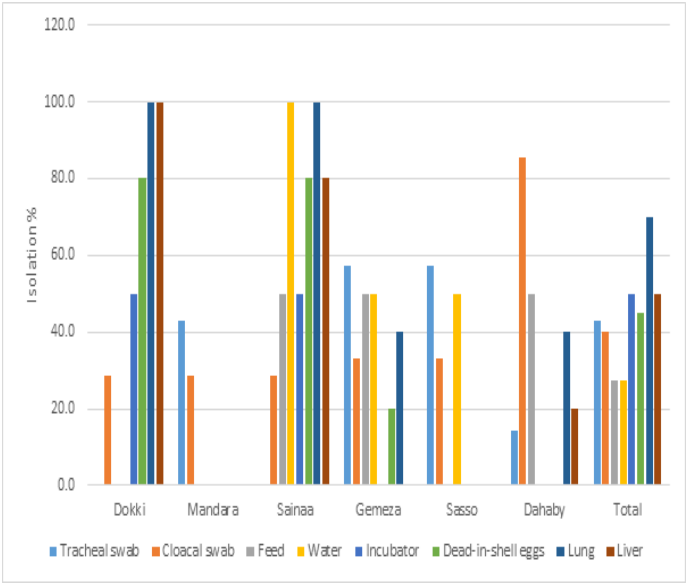


Figure 2. Fungal isolation rates from breeder layers, dead-in-shell eggs and hatched chick's organ samples

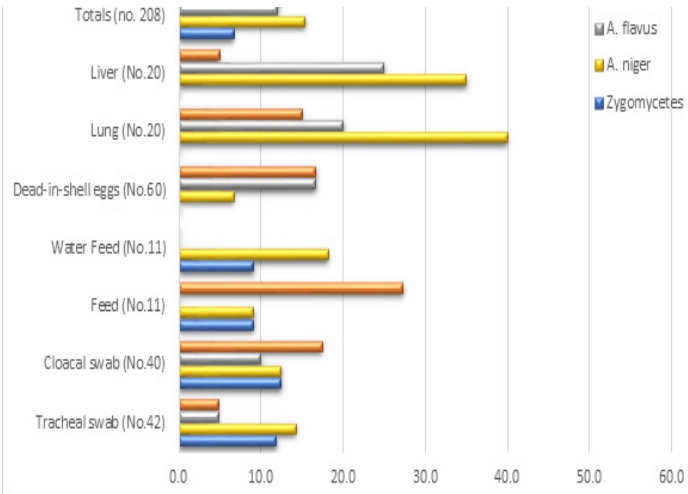


Figure 3. Macroscopical and microscopical appearance of isolated A. flavus (A-C), A. terreus (D-F), and A. niger (G-I)

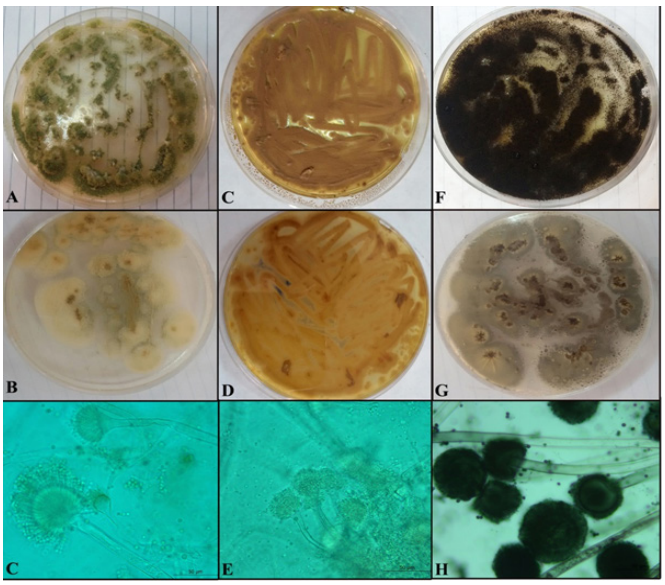
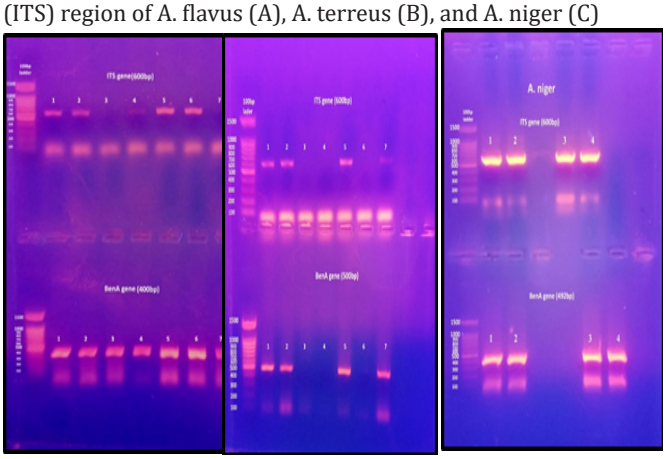


Figure 4. The PCR amplification of the internal transcribed spacer (ITS) region of A. flavus (A), A. terreus (B), and A. niger (C)



A. A. flavus; lanes (1-4) isolates from Dokki, Sainaa, Dahaby, Sasso breeders, respectively. Lanes (5-7) isolates from Dokki dead-in-shell embryos, Dokki chick's liver, and Sainaa chick's lung

B. A. terreus; lanes (1-4) isolates from Dokki, Dahaby, Mandara, Sainaa breeders, respectively. Lanes (5-7) isolates from Dokki dead-in-shell embryos, Dahaby dead-in-shell embryos and Dokki chick's lung, respectively

C. A. niger; lanes (1&2) isolates from Sainaa and Dokki breeder chickens, respectively. Lanes (3&4) isolates from Sainaa dead-in-shell embryos and hatched chick's lung, respectively.

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