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Research Paper

DETECTION OF aspHS GENE IN ASPERGILLUS FUMIGATUS DURING ASPERGILLUS INFECTION IN POULTRY AND HUMAN CONTACT

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Aspergillosis has a significant health concern for poultry and human, it causes economic losses in poultry flocks and many pulmonary infections in human. So our study was conducted to detect the incidence of *Aspergillus* species in both chicks and farm workers in private poultry farm in Qena Governorate, Egypt. 29 out of 40 examined laryngeal swaps (72.5%) collected from chicks and 15 out of 40 sputum samples (37.5%) collected from farm workers were positive for *Aspergillus* species. *A.flavus*, *A.fumigatus* and *A.niger* were detected in 60, 10 and 30% of examined chicks samples, while 12.5, 15 and 30% of examined workers samples were positive for these species respectively, beside *A.ochraceus* which was isolated at a low percentage (5%). *aspHS* gene was detected in 75 and 33.3% of *A.fumigatus* strains isolated from chicks and workers respectively. The sequence of the detected *aspHS* gene in our isolates was similar to reference strains registered with Gene bank at the percentages of 100% and 99.4%. The zoonotic role of this gene during infection was reinforced after the identity was found between our isolates in the amplified part (isolate No. 2 obtained from chicks and isolate No. 9 obtained from workers).

Keywords: Chicks, Workers, Aspergillosis, A.fumigatus, aspHS gene

INTRODUCTION

Aspergillosis is a common sapromycotic zoonotic disease of birds and man and has been emerged as an important respiratory infection due to the extensive use of antibiotics and immuno suppressive agents (Biswas *et al*., 2010), it caused by genus *Aspergillus*, which involving about 200 species, these species were environmentally distributed in outdoor and indoor air (Latgé, 2001). *Aspergillus fumigatus* is the prime causative agent of Invasive Aspergillosis (IA) especially in immunocompromised hosts,

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with a mortality rate higher than 50% followed by *Aspergillus flavus*, *Aspergillus niger*, and *Aspergillus terreus* (Oliveira and Caramalho, 2014).

Conidia of genus *Aspergillus* may persist in the atmosphere, soil and water for prolonged time because of the nature of the cell wall which protects them from several physical and chemical stresses (Araujo *et al*., 2006; and Latgé, 2007). In birds, acute Aspergillosis includes nonspecific clinical signs as anorexia, lethargy, ruffled feathers, respiratory signs, polyuria, stunting or sudden death (Milos *et al*., 2011).

Inhalation of several hundreds of conidia during routine daily activities considered the main route of human infection (Latgé, 2001). Certainly, poultry farm workers are at high risk of developing respiratory problems as they are exposed to high levels of dust containing *Aspergillus* spores during handling and processing of contaminated materials (Sabino *et al*., 2012). According to the state of the host immunity system; alveolar macrophages can detect the inhaled conidia, engulf and destroy them or severe allergic reactions associated with systemic disorders may occur especially in immunocompromised patients (Brakhage *et al*., 2010; and Milos *et al*., 2011).

Identification of *Aspergillus* species has historically been based on traditional diagnostic methods such as histopathology and culture, which are still considered the gold standards because of their simplicity and accessibility. Indeed, novel serologic and molecular techniques have been developed to confirm the isolates, detect the virulence related genes and to obtain an early diagnosis (Balajee *et al*., 2009).

Aspergillus fumigatus has certain virulence factors associated with its structure, capacity for growth, adaptation to stress conditions, mechanisms for evading the immune system and its ability to cause damage to the host. These factors include *abr1*, *alb1*, *arp1*, *aspHS* and *catA* (Abad *et al*., 2010).

The *aspHS* gene attracted our interest as it is a novel target for the specific detection of *A.fumigatus* by PCR and because of its possible cytotoxic effect and hemolytic activity. So our study aimed to detect the incidence of *Aspergillus* species especially *A.fumigatus* through detecting *aspHS* as a common virulence gene during respiratory infection in poultry and human.

MATERIALS AND METHODS

Study Design and Duration

Samples were collected from poultry and poultry farm workers in Qena Governorate during the period from January to June 2016.

Samples Collection

Poultry Samples

Laryngeal swaps were collected from 40 chicks in private poultry farm in Qena at age of 2-3 weeks suffered from respiratory distress, dyspnea, gasping and nasal discharge. Postmortem examination of dead cases revealed congested lungs with grayish white nodules and frothy exudate. Swaps were put in sterile screw-capped tubes containing 5 ml Sabouraud dextrose broth and transported to the microbiology laboratory for mycological examination.

Workers Samples

Forty sputum samples were collected from workers in the poultry farm (21-40 years old), showed signs of a cough, chest pain and difficult breathing. The samples were transported in screw-capped tubes containing 5 ml Sabouraud

dextrose broth and transported to the microbiology laboratory for mycological examination.

Isolation and Identification of *Aspergillus* Species

Inoculated test tubes were incubated at 25 °C for 5 days. A loopful from each incubated tube was plated on the surface of Sabouraud Dextrose Agar (SDA) containing 0.05 mg of chloramphenicol per ml by using streaking technique method. The plates were incubated at 25 °C for 5-7 days (Forbes *et al*., 2014). The mold isolates were identified according to Moubasher (1993), Klich (2006), Domeish *et al*. (2007) and Ellis *et al*. (2007). The Identification depended on macroscopic identification (culture characteristics; colony color, shape and texture) and microscopic identification for conidia (conidia shape, phialide shape and branching pattern).

Extraction of DNA from *A.fumigatus* Isolates

Fungal mycelium and spores which obtained from each sample were cultured in a flask containing 50 ml potato dextrose broth for 5 days. The Broth was filtrated, mycelium and spores were used for DNA extraction by different weights (3-15 mg). They were broken down by using Ready Prep Mini-grinders (Bio-Rad) with liquid nitrogen. Then 500 µl of lysis buffer containing 40 µg/ml of RNAseA (Fermentas, St. Leon-Rot, Germany) was added and the mixture was incubated at room temperature for 10 min. For DNA purification, 150 µl of potassium acetate was added to the mixture and centrifuged for 5 min at 8,000 g. The supernatant was recovered, transferred into a clean tube and centrifuged as before. The DNA was precipitated with 600 µl of ice-cold isopropanol and 300 µl of ice-cold ethanol in consecutive steps, followed by centrifugation at 14,000 g for 15 min and finally reconstituted in 50 µl of PCR-grade water (Bioline, London, UK). DNA concentration and quality were measured with a Nano Photometer (IMPLEN, Schatzbogen, Germany) and the extracted DNA was stored at -80 °C (Liu *et al*., 2000).

PCR Amplification and Nucleotide Sequencing

PCR protocol used in this study was performed according to Dennis Lo *et al.* (2006) in an MJ Mini Personal Thermal Cycler (Bio-Rad) in a 25 µl volume containing 3.2 µM of each primer (Table 1), 0.2 mM of total dNTP, 2.5 mM of magnesium chloride, 0.03 U of Taq DNA polymerase (Bioline) and 1 µl of DNA template (10 ng/µl). The amplification conditions used were: an initial denaturation at 95 °C for 10 min followed by 32 cycles of 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, and a final step at 72 °C for 10 min. Amplicons were analyzed by 2.5% agarose gel electrophoresis with Gel Red Nucleid Acid Stain (Biotium, Hayward, CA, USA) and visualized using a U: Genius Gel Imaging System (Syngene, Cambridge, UK). Samples included DNA of positive control and negative control. Purified PCR products were sequenced using a prism Big Dye terminator V3.1 Kit (applied Bio system Cat. No. 4336917) on DNA automated sequencer (applied Bio systems). Two isolates (one from chicks and the other from workers) were analyzed in forward and reverse directions for sequence of *aspHS* gene. Sequence alignment was performed using multiple alignment algorithms in megalign (DNASTAR, Window version 3.12e).

Table 1: aspHS Primer Sequence

Primer	Sequence	Target Site	Amplicon
F-*asphs*	5-tggtacaaggacggtgacaa-3	168-187	180-bp
R-*asphs*	5-gtcccagtggactcttccaa-3	328-347	

Phylogenetic Analysis

Phylogenetic tree was based on *aspHS* gene nucleotides sequence which was performed on our two *A.fumigatus* isolates to investigate the identity of amplified fragment of *aspHS* gene in our isolates with *A.fumigatus* reference strains registered with Gene bank using MEGA version 2.1 (Kumar *et al*.,2001).

RESULTS

The incidence of Aspergillosis was calculated in forty chicks suffered from respiratory disorders. *Aspergillus* species were detected in 72.5% (29/40) of the examined laryngeal swabs inoculated on SDA media (Figure 1). These cases were selected on the basis of clinical manifestations and postmortem findings. On the other hand, *Aspergillus* species were isolated at percentage of 37.5% (15/40) from sputum samples collected from the farm workers with a case history of recurrent respiratory manifestations (Figure 1).

Data postulated in Table 2 showed that *A.flavus, A. fumigatus* and *A. niger* were detected in examined chicks samples at the percentages of 60, 10 and 30% respectively, while the same species were detected in workers samples at the percentages of 12.5, 15 and 30% respectively. *A. ochraceus* was detected in 5% of the examined workers samples only.

Figure 1: Incidence of Aspergillus species in the Examined Chicks and Farm Workers Samples

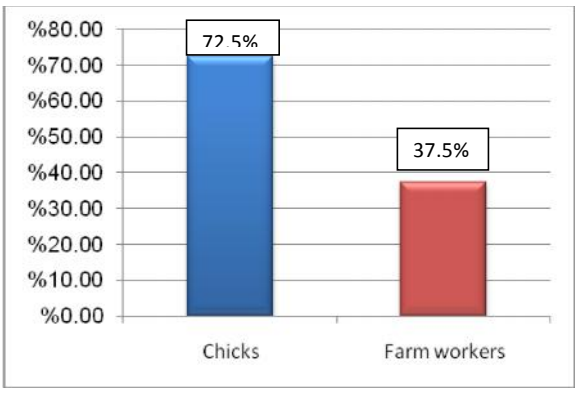


Table 2: Incidence of Aspergillus Species in Chicks and Farm Workers Samples

Aspergillus spp.	Chicks Samples (No = 40)		Workers Samples (No = 40)	
	No. of Positive Samples	%	No. of Positive Samples	%
A. flavus	24	60	5	12.5
A. fumigatus	4	10	6	15
A. niger	12	30	12	30
A.ochraceus	0	0	2	5

Table 3: Occurrence of aspHS gene in A.fumigatus Strains Isolated from Chicks and Farm Workers Samples

No. of *A.fumigatus* Isolates	Source	*aspHS* gene
A.fumigatus (1)	Chicks	+
A.fumigatus (2)	Chicks	+
A.fumigatus (3)	Chicks	-
A.fumigatus (4)	Chicks	+
A.fumigatus (5)	Workers	-
A.fumigatus (6)	Workers	+
A.fumigatus (7)	Workers	-
A.fumigatus (8)	Workers	-
A.fumigatus (9)	Workers	+
A.fumigatus (10)	Workers	-

In the present study *aspHS* gene was amplified in 75% (3/4) of *A.fumigatus* strains isolated from chicks samples and 33.3% (2/6) of *A.fumigatus* strains isolated from workers samples (Table 3 and Figure 2).

The result of *aspHS* gene sequence for our two *A.fumigatus* isolates (Table 4) when compared with gene sequences of reference isolates registered with Gene bank using the

Figure 2: PCR Amplification Profile Showing the Presence of aspHS Gene of A.fumigatus Strains Isolated from Chicks and Farm Workers, M: Marker (100 bp Molecular) Lane 1: Positive Control, Lane 2, 4, 6, 8, 9 Band Size Positive for aspHS Gene 180 bp) and Lane 10: Negative Control

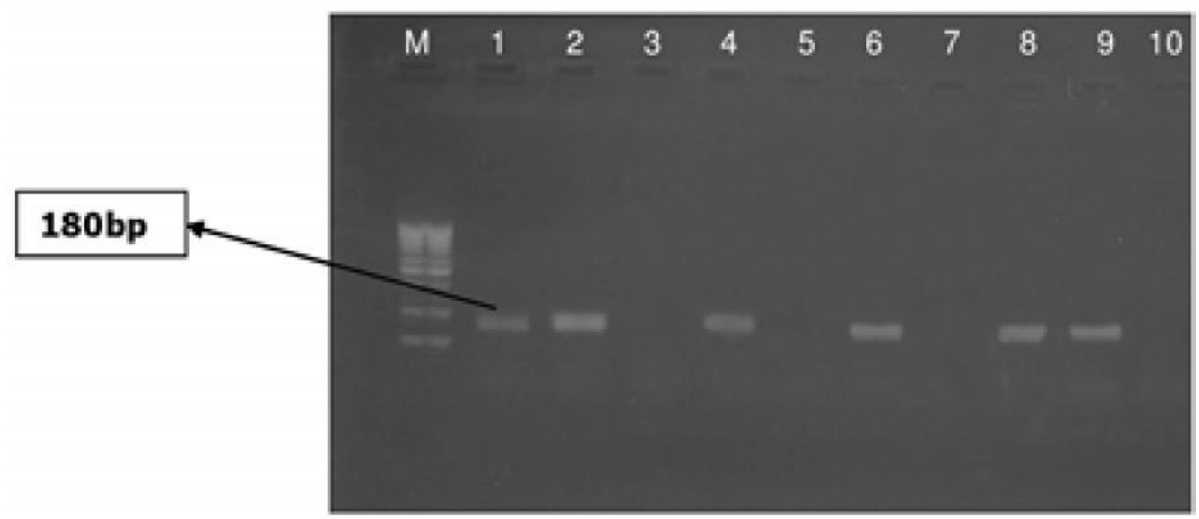
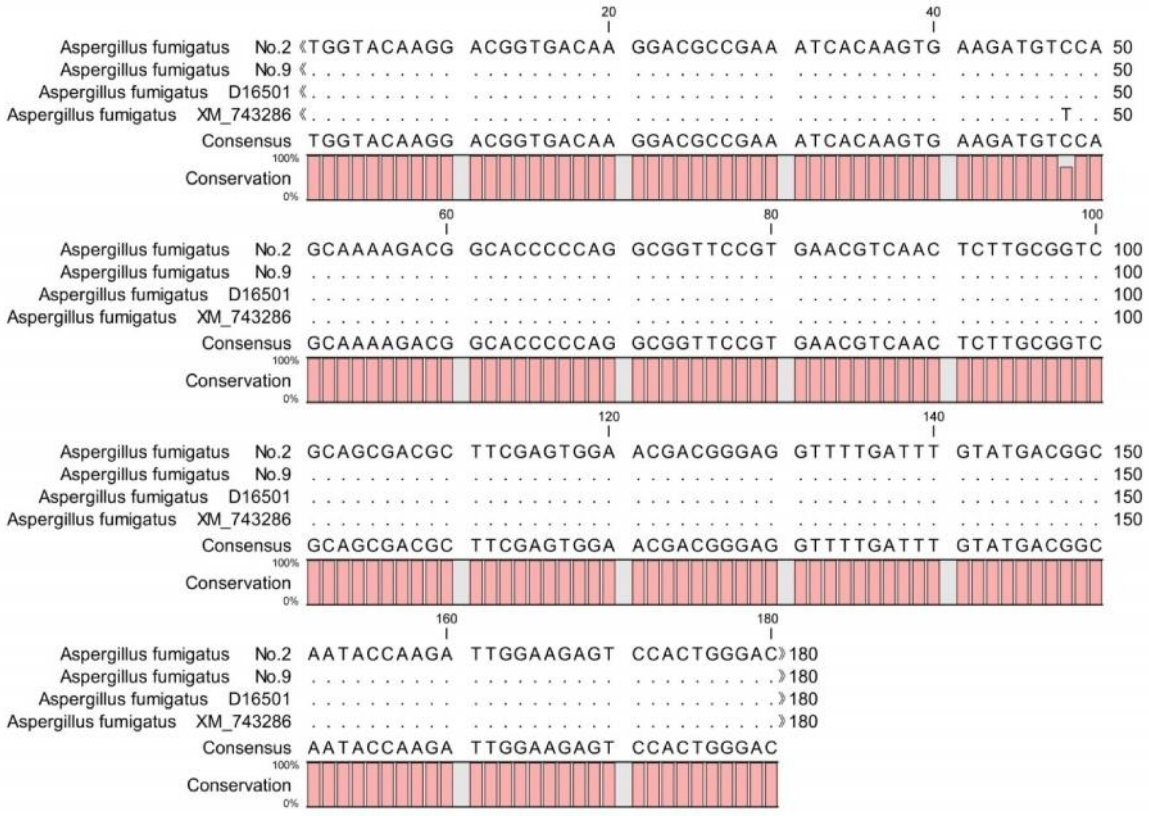


Figure 3: Alignment of aspHS Gene of Our A.fumigatus Isolates Compared with Reference Strains Retrieved from Gene Bank



Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that the identical results were found to be 100% with *A.fumigatus* No. D16501 while it showed 99.4% identity and 0.6% divergence with *A.fumigatus* No. XM-743286. Our isolates were shown 100% identity with each other in the amplified fragment (180 bp) (Figure 3).

The phylogenetic tree by using nucleotide sequence confirmed the identity of sequence alignment results of *aspHS* in our isolates with *A.fumigatus* strain No. D16501 and *A.fumigatus* No. XM-743286 and it confirm the identity of our isolates with each other in amplified fragment (isolate No. 2 and isolate No. 9) (Table 4 and Figure 4).

DISCUSSION

In the current study it was observed that *Aspergillus* species were detected in 72.5% of chicks suffering from severe respiratory signs and a high mortality rate at age 2-3 weeks during the winter season (Figure 1), lower incidences were reported by Sajid *et al*. (2006) and Salem and Ali (2014), the difference can be accepted due to several factors as the density of birds, topographic conditions and other ambient conditions (Sabino *et al*., 2012). Similar findings were recorded by Richard *et al.* (1991) and Bhattacharya (2003), who observed similar clinical signs and mortality rate in infected birds. Bennett *et al.* (1988) reported that phagocytes immaturity and environmental factors may increase susceptibility of young chicks to *Aspergillus* infection. These results agree with Kunkle and Rimler (1996) who noticed that the mortalities are more common in winter when indoor gas levels tend to be highest.

The fungal contamination of poultry farms may occur through use of a moldy litter, poor quality feedstuffs or through inappropriate bedding management (Santos *et al*.,1996). Also, humidity and temperature conditions in poultry farms increased the growth of hyphae and a sexual multiplication of airborne conidia, which are subsequently disseminated and inhaled by the birds. These findings denote the role of poultry in human Aspergillosis (Latgé, 2001).

From the public health point of view, Aspergillosis has been incriminated as one among the most feared opportunistic infections in human because it is capable of causing several distinct pulmonary diseases (Krel *et al.*, 2014). The incidence of *Aspergillus* species in examined sputum samples was 37.5% (Figure 1) which collected from workers with respiratory affections

Table 4: Accession Number of A.fumigatus Strains Isolated from Chicks and Farm Workers

Isolate Number	Sources	Accession Number
A.fumigatus No. 2	Chicks	MF537632
A.fumigatus No. 9	Farm workers	MF537633

Figure 4: Phylogenetic Tree of the Entire Nucleotide Sequence of aspHS Gene of Our A.fumigatus Isolates Compared with Reference Strains Retrieved from Gene Bank

at age of 21-40 years old which is the actual age of work. Lower incidences were obtained by Pursell and Paredes (1990) and Shrimali *et al*. (2013) who detected *Aspergillus* species in 4.8%, 29% of the examined sputum samples respectively. While a higher percentage (44.7%) were obtained by Al-Malaky *et al*. (2015). This discrepancy may be due to the population growth, extensive use of antimicrobial agents, virulence of fungi and personal hygiene (Schraufnagel, 2009).

In the present study, several *Aspergillus* species such as *A.flavus, A.fumigatus and A.niger* were isolated from infected chicks and worker samples (Table 2). Several authors found that these species are the most causative agents in the pulmonary infections (Njunda *et al*., 2012; Barberan *et al*., 2012; Shrimali *et al*., 2013; Zaki *et al*., 2016; and Sajid *et al*., 2006). Although *A.fumigatus* wasn't isolated by a higher incidence than the other species in this study but it considered a highly pathogenic species and a main etiological agent of Aspergillosis due to the smaller size of *A.fumigatus* spores than those of other *Aspergillus* species, so it can easily be inhaled, also *A.fumigatus* produces different virulence factors such as haemolysins, proteolytic enzymes and different toxins and these explain the high mortalities associated with invasive Aspergillosis (Akan *et al*., 2002; Summerbell, 2003; Chamilos *et al*., 2006; Khosravi *et al*., 2008; and del Palacio *et al*., 2008).

According to many investigators who showed the important role of *Aspergillus* section *Fumigati*e especially *A.fumigatus* in Aspergillosis, *aspHS* gene was selected in this study as a specific target for detection of *A.fumigatus* during infection. This virulence gene encodes a haemolysin, which is over expressed *in vivo* during infection and confirmed the pathogenic role of *A.fumigatus* in Aspergillosis (DE-Cerio *et al*., 2013).

PCR results revealed that *aspHS* gene was detected in *A.fumigatus* strains isolated from chicks and farm workers (Table 3) and the sequence alignment of *aspHS* gene in our isolates has a high similarity with *A.fumigatus* strains registered with gene bank, these results were similar to DE-Cerio *et al*. (2013) who detected *aspHS* genes in most of *A.fumigatus* strains isolated from infected individuals and found that their isolates have identity with *A.fumigatus* reference strains registered with gene bank.

Our isolates showed 100% identity with each other in amplified fragment of *aspHS*gene. This similarity may be due to the collection of samples from the same environment (poultry farm), at the same time and the hosts are highly contacted to each other. These results were in line with Dhama *et al*. (2012) who found that birds, as well as people who work with them get affected after inhaling these spores in contaminated feed or litter.

CONCLUSION

From our study, it can be concluded that poultry and human can share *Aspergillus* infection. Presence of *Aspegillus* species in poultry farms causes great problem among occupations, so we recommend with taking special precautions as wearing gloves and masks during handling of birds, avoidance of damp litter and dust by adequate ventilation in poultry farms and moldy feed shouldn't be given to birds; all these may decrease the occurrence of infection. In case of *Aspergillus* infection, we should perform a rapid molecular diagnosis, treatment of infected cases and hygienic condemnation of dead chicks to decrease spreading of infection.

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