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Hydrolytic Enzymes as Probable Virulence Factors For *Aspergillus ochraceus* Fm90 in Aspergillosis

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THE MAIN concept of this study was to assess the potentiality of hydrolytic enzymes as virulence factors for *Aspergillus* species during aspergillosis infection process. Forty *Aspergillus* species were isolated from medical (15 isolates) as well as environmental isolates (25 isolates) from soils, outdoor and indoor environments. Extracellular proteases, phospholipases and esterase activities were measured. The pathogenicity of some *Aspergillus* species was *in vivo* assessed. It was represented in mean survival times, mortality percentages, fungal counts in different organs, and histopathological examination of lung tissue. The expression levels of protease, phospholipase and esterase genes were studied by real time PCR. Four physiologically significant isolates were selected out of forty and were identified up to molecular level. *Aspergillus ochraceus* fm90 recorded the highest pathogenicity as represented by mortality percentages and mean survival times of mice, while *A. flavus* fm90 was the least pathogenic one. Expression of genes of protease, phospholipase and esterase was found to be greater in *A. ochraceus* fm90 than in *A. flavus* fm90. It can be concluded that pathogenicity is probably related to physiological (enzymatic) activities of the isolates. Also, variation in expression levels of protease and phospholipase genes in *A. ochraceus* fm90 and *A. flavus* fm90 could denote their possible involvement in the pathogenicity process.

Keywords: *Aspergillus*, Aspergillosis, Enzymes, Murine model of infection, Gene expression.

Introduction

Aspergilli are common soil fungi found in great numbers in dust and decomposing organic matter and release huge amounts of spores which comprise 25% of total airborne fungi in the atmosphere (Raper & Fennell, 1965). They are opportunistic molds responsible for aspergillosis which is a variety of infections with different conditions. These displays comprise diseases that range from allergic responses to the organism (allergic bronchopulmonary aspergillosis), to colonization with *Aspergillus* spp. (aspergilloma or fungus ball) and invasive infection (Kauffman et al., 2011). A greater number of patients are at risk for invasive aspergillosis leading to the increased number of cases of this disease. Human immune deficiency syndrome patients, those with hematologic malignancies, such as acute myelogenous leukemia, and patients undergoing allogeneic hematopoietic stem cell transplantation are at highest risk for developing invasive infection (Walsh et al., 2008 and Patterson et al., 2000).

To understand the pathobiology of *Aspergillus,* this would need analysis of the putative fungal virulence factors that enhance growth and/or survival in the lung environment, as well as the awareness of the immune factors containing *Aspergillus* in the immunocompetent host which can be weakened by immunosuppressive treatments, triggering invasive aspergillosis (Latgé, 2001). Depending on the immunologic status of the host, inhalation of *Aspergillus* conidia can initiate different clinical aspergillosis forms (Xess et al., 2004). There is no distinctive key virulence factor for the progress of this fungus in the patient and its virulence is polygenetically controlled. There is a group of molecules and genes that were reported to be related to the virulence of this fungus. This includes toxins (fumagilin, helvolic acid, and Asp-haemolysin), and enzymatic proteins as alkaline serine proteases (Alp and Alp2), dipeptidyl-peptidases (Dpp IV and Dpp V), phospholipase B (Plb1 and Plb2) and phospholipase C (Rementeria et al., 2005).

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The present study aimed to isolate Aspergilli from various environmental habitats as well as from patients. The aim was extended to measure the extracellular phospholipase, esterase, and protease activities. In addition, the pathogenicity of some *Aspergillus* species was assessed *in vivo*. Moreover, the expression levels of genes representing the studied enzymes were evaluated.

Materials and Methods

Environmental isolates

Soil samples from different localities (Dar El-Salam (Cairo), Zamalek area (Cairo), Giza Zoo (Giza), Kasr El-Einy Hospital (Cairo), and Cairo University (Giza)) were collected and cultured on Czapek-Dox agar medium (g/L): Sucrose, 20; $NaNO_3$, 2; K_2HPO_4, 1; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01 and agar, 20. Streptomycin (30µg/ml) was added to the above medium after sterilization and cooling using soil dilution plate method (Johnson et al., 1960). Air samples were collected from indoor and outdoor environments of buildings suspected with fungal contamination, such as Microbiology Laboratory in Kasr- Eleiny Hospital (Cairo, Egypt) and from an administrative office at Zamalek area (Cairo, Egypt) at which fungal deterioration of some building materials occurred associated with health problems to some of the immunocompromised building occupants. Indoor samples were collected from settled dust and building materials samples on sterile cotton swabs from desks, deteriorated wooden floor and air conditioner's filter inside the administrative office at Zamalek area. Air borne Aspergilli were isolated using settle plate method adopted by Hoekstra et al. (2000). Swabs samples were cultured on plates containing sterile Sabaroud's agar medium (which contained (g/L): Sucrose, 20; peptone, 10 and agar, 20). All plates were incubated for 5 days at 30°C to be isolated, purified and identified. Plastic plates (9cm diameter) containing sterile Sabaroud's agar media are placed out door (outside the window) with covers open for one hour, plates were then closed and incubation was performed for 5 days at 30°C for further isolation, purification and identification.

Clinical isolates

Clinical samples culture from patients admitted to Kasr- Eleiny Hospitals, Cairo University suffering from *Aspergillus* infection (invasive aspergillosis). Samples were sputum, endotracheal aspirate and tissue specimens. Tissue specimens were saved in sterile 0.9% NaCl saline if transport is being delayed. Samples from other body sites (e.g.; nail, body fluid, discharge …etc.) were also collected under sterile conditions for further culturing and examination.

The developed fungal colonies identification up to the species level was accomplished according to morphological characters and microscopic examination (Raper & Fennell, 1965).

Enzymatic activities of the isolated Aspergillus species

Phospholipases

Phospholipase activity assessment was done for the studied isolates using plate method (Price et al., 1982), with modifications. Modified Sabroud-Dextrose Agar Medium (g/L): Glucose, 40; peptone, 10; NaCl, 58.4; $CaCl_2$, 5.5 and agar, 20 was prepared, and then autoclaved at 121°C and 1.5 bars for 20min. Sterile egg yolk was centrifuged at 5000rpm for 30min, after which 20ml of supernatant was added to the cooled medium and dispensed into 9cm plates. Using sterile spore buffer, a spore suspension (10^8 spore/ml) was prepared from 7 days old culture for each of the test isolates. Each plate is then inoculated with the spore suspension of the test isolate in a pore (1cm diameter) made at the center of the plate using sterile cork borer and incubated for 5 days at 30° C. then they were examined for the formation of precipitation zone around the colonies. A halo precipitant surrounding the colonies was measured on day five, the ratio of colony diameter to colony diameter plus precipitation zone and expressed as (PA) value was used to evaluated phospholipase activity:

$$PA = \frac{\text{Fungal colony diameter}}{\text{Fungal colony diameter} + \text{Precipitation zone}}$$

For induction of extracellular esterase secretion, yeast extract peptone glucose (YPG) broth medium (g/L): Glucose, 20; peptone, 20; yeast extract, was used after sterilization by autoclaving at 121°C and 1.5 bars for 20min. After addition of the organisms spore suspension (10^8spore/ml) to a flask containing 100ml of growth medium, shaken at 150rpm at 30°C for 5 days and at the end of incubation period, filtration is done using Whatmann filter paper and the filtrate is used to test for extracellular esterase activity.

Esterase activity was measured colorimetrically using 1-naphthylacetate as substrate. The fungal filtrate (2ml) was incubated with 0.5ml of 1.06mM substrate and 2ml of 10mM phosphate buffer saline (PBS), pH 7.5, at 30°C for 90min. The reaction termination was done by the addition of 0.5ml of sodium dodecyl sulfate solution (10%) and 0.5ml of 0.2% fast blue R.R salt solution were added. Absorbance was read at 540nm 20min after adding the dye. Under the assay conditions, Specific esterase activity (mg 1-naphyhol released/ml of fungal filtrate/hour) was calculated using 1-naphthol standard curve (Khosravi et al., 2008).

Proteases

For the induction of protease secretion modified Czapek-Dox medium (g/L): Sucrose, 30; casien, 10; K_2HPO_4, 1; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01 was used after sterilization by autoclaving at 1.5 bars and 121°C for 20min. After inoculation of each organism (10^8spore/ml) to a flask containing 100ml of growth medium, the flask was shaken at 150rpm at 30°C for 5 days. At the end of incubation, filtration was done using Whattman filter paper and the filtrate is used to test for extracellular protease activity (Coral et al., 2003).

The protease activity was assayed according to Lovrien et al. (1985) with little modification. The reaction mixture containing 0.5ml of 1% (w/v) casein in Tris buffer (pH 7.5) and 50µl of enzyme was incubated at 40°C for 30min. The reaction was stopped by addition of 12% (w/v) trichloroacetic acid (TCA) and the precipitate was removed by centrifugation at 10,000rpm for 5min (Coral et al., 2003). Amino acids liberated from casein hydrolysis are then estimated using ninhydrin (Kendall, 1963).

Specific proteases activity (mg amino acids released/ml of fungal filtrate/hr) was calculated using glycine standard curve.

Molecular identification of Aspergillus isolates

Aspergillus species identification was then verified using nuclear ribosomal DNA internal transcribed spacer (ITS) sequencing. Genomic DNA was isolated using the protocol of GeneJet Plant genomic DNA purification Kit (Thermo) # K0791 **(**http://www.thermoscientificbio.com/). Internal transcribed spacer (ITS) region of 5.8S rRNA was amplified using the primers ITS1 (5´TCC GTA GGT GAA CCT TGC GG 3´) and ITS4 (5´TCC TCC GCT TAT TGA TAT GC 3´). Sequencing of PCR amplified product was performed at GATC Company (Germany). The resulting sequence was entered into the BLAST algorithm of (NCBI) National Centre of Biological Information database for obtaining phylogenetic sequences which are closely related. MEGA 6 software was used to construct phylogenetic trees. Sequence which was obtained then submitted to the GenBank of (NCBI) National center for biotechnological information database. For each isolate, a strain identifier and an accession number were given.

Experimental aspergillosis in mice

Pathogenicity of both the statistically highest and lowest enzymatically active species selected from both environmental and medical sources was *in vivo* assessed by murine model of infection.

The experimental design was approved by The Institutional Animal Care and Use Committee (IACUC) at Faculty of Science, Cairo University, which is organized and operated according to the World Organization for Animal Health (OIE) and Guide for the Care and Use of Laboratory Animals 8th edition 2011 and was given an IACUC Permit Number: CUFS, F, Microbiology, 47,1.

Fifty female albino mice weighing 18 to 20g were housed in ten groups (5 mice in each group) into the cages. Under hygienic conditions, the mice were supplied with sterile drinking water containing 500µg/ml chloramphenicol.

Infection by test isolates of aspergilli

Immunosuppression was done by subcutaneous injection of 5mg hydrocortisone acetate on days -4, -2, 0, 2 and 4 of infection.

Control groups (1&2) were injected by sterile saline solution (0.85% NaCl).

One ml (10^8spores/ml) of spore suspension prepared from 5 days old culture of the selected isolate on Sabroud's medium using sterile spore buffer was injected in mice *via* intraperitoneal route (Chhabra & Dhakad, 2008).

Ten groups were designed as follows:

Group 1: Negative control (normal mice injected with saline).

Group 2: Negative control (immunosuppressed mice injected with saline).

Groups 3, 5, 7 and 9: Normal mice infected with the most enzymatically active medical isolate, the least enzymatically active medical isolate, the most enzymatically active environmental isolate and the least enzymatically active environmental isolate, respectively.

Groups 4, 6, 8 and 10: Immunosuppressed mice infected with the most enzymatically active medical isolate, the least enzymatically active medical isolate, the most enzymatically active environmental isolate and the least enzymatically active environmental isolate, respectively.

Survival times and Mortality percentages of infected mice were monitored. Mice were observed daily for 4 weeks post inoculation. Dead mice were dissected and their lungs (left), livers and kidneys were removed. The number of colony forming unit (CFU) in various organs was determined by homogenizing in phosphate buffer saline pH 7 and plating 0.1 ml on 9cm Petri dish containing sterile Sabouraud dextrose agar then incubated at 30ºC (Khosravi et al., 2012). After incubation, colonies were counted and expressed as CFU/corresponding mean survival time.

The lungs (right) were fixed in 10% formalin. Sections were stained with hematoxylin and eosin to observe damage of tissue (Kothary et al., 1984).

Gene expression assessment by real time PCR

The rate of gene expression can be quantitatively detected by measuring the amount of RNA by real time PCR. Forward and reverse primers were designed for amplification of protease (alkaline serine protease, PR1), phospholipase (lysophospholipase, Plb1), and esterase (lipase/serine esterase) genes using PCR. Designing primers was performed using the software DNAstar. Table 1 lists the sequences of these primers.

RNA was extracted using TRIsure™ RNA extraction kits (http://www.bioline.com/). Using RNA template, cDNA synthesized using SensiFAST™ cDNA Synthesis Kit (http://www.bioline.com/). Conventional PCR was used to ensure that all primers were specific for the selected genes. Electrophoresis of PCR product was performed in 1.5% agarose gel for 1.5hr at 7.0V/cm^2 and stained by 0.5g/ml ethidium bromide. SensiFAST™ SYBR® No-ROX One-Step Kit (http://www.bioline.com/) was used to perform real time PCR. The obtained Regression curves were used in comparing gene expression levels in the isolates tested.

Statistical analysis

The data presented were means of three replicates. The SPSS 20 software was used in determination of least significant difference (LSD).

LSD was calculated using One-Way ANOVA: Post Hoc Multiple comparisons at 99% confidence interval (probability of error P= 0.01). Standard error was given.

The Duncan method then displayed means for groups in homogenous subsets, where means followed by the same letters are non-significantly different from one another while means which are followed by different letters are considered significantly different from one another.

TABLE 1. PCR primers sequences of protease, phospholipase and esterase genes and their properties.

Gene		Sequence	Expected PCR product length (bp)	Annealing temp ºC	G-C ratio %
Esterase	Forward	GATCCGGCCTACCTATCAAAC	181	51.2	52.38
	Reverse	AGAATTGAGCTGGCGTTAGAG	181	51.2	47.62
Protease	Forward	TGCTCCTGGTGTGGACATTC	143	54.1	55
	Reverse	CTTGACCTCCAGCCAGAGTC	143	54.1	55
Phospolipase	Forward	ACCCGTTGATTCAGCCAGAG	150	54.0	55
	Reverse	AGGAAGTACCGTTGGCGATG	150	54.0	55

Results

Sample collection and isolation

A total of 40 *Aspergillus* isolates were obtained, (15 clinical and 25 environmental. The 15 clinical isolates were isolated from fifteen patients with aspergillosis, while the 25 environmental isolates were isolated from soils at different sites (16 isolates), out and indoor environments (9 isolates).

Screening for extracellular enzymatic activities

Screening for extracellular phospholipase activity

In the current study, it was noticed (Table 2) that 21 isolates (52.5%) of the total 40 isolates were phospholipase-positive. Maximum phospholipase production was achieved by *Aspergillus* species isolated from Aspergillosis patients; 11 isolates out of 15 isolates (73.3%). For environmental isolates, 6 isolates of *Aspergillus* species isolated from soil out of 16 isolates (37.5%) were phospholipase-positive positive. While for outdoor and indoor isolates, 4 isolates representing 44.4% of the total environmental isolates (9) were phospholipase-positive.

Screening for extracellular esterase activity

In this study, Table 2 reveals that positivity of esterase activity was found in 34 isolates of the total 40 isolates (85%). maximum esterase occurrence was noticed for *Aspergillus* species isolated from aspergillosis patients; 14 isolates out of 15 isolates (93.3%). For *Aspergillus* species isolated from soil, 12 isolates out of 16 isolates (75%) were esterase positive, while for out and indoor isolates, 8 out of 9 isolates (88.8%) were esterase positive.

Screening for extracellular protease activity

Protease enzyme was positive in a total of 27 out of 40 isolates (67.5%); 13 out of 15 clinical isolates (86.7%), 7 out of 16 isolates (43.8%) from soil and in 7 out of 9 isolates (77.78%) from out and indoor isolates (Table 2).

Molecular identification of the selected Aspergillus species

Both the statistically highest and lowest enzymatically active isolates were selected from both environmental species and those isolated from patients for further characterization such as molecular identification, assessment of pathogenicity *in vivo* and testing gene expression of some pathogenicity related genes.

Between The most enzymztically active isolates, *Aspergillus ochraceus* isolated from patient no. 14 (from medical source) and *Aspergillus oryzae* isolated from a wooden desk in an office with fungal infection outbreak (from environmental source) were selected.

Aspergillus flavus isolated from patient no. 15 (from medical source) and *Aspergillus terreus* isolated from Cairo University soil (from environmental source) were selected from the least enzymatically active isolates.

Identification of the four selected *Aspergillus* species was further confirmed using nuclear ribosomal DNA internal transcribed spacer (ITS) sequencing. The PCR products were 541, 481, 481 and 541 bp for *A. ochraceus*, *A. flavus*, *A. terreus* and *A. oryzae,* respectively. The traditional Sanger technology and the new 454 technology were combined for sequencing the PCR products. The obtained nucleotide sequence was deposited in NCBI GenBank and were each given a strain identifier, *A. ochraceus* fm90, *A. flavus* fm90, *A. terreus* fm90, and *A. oryzae* fm90 with accession numbers MF284669, MF284670, MF284671, and MF284672, respectively. The phylogenetic tree was constructed (Fig. 1) to show sequence alignment with available sequences from NCBI data bank

In vivo assessment of Aspergillus species pathogenicity

In the present study, the experimental design was approved by the Institutional Animal Care and Use Committee (IACUC) at Faculty of Science, Cairo University and was given an IACUC Permit Number: CUFS, F, Microbiology, 47, 15.

It was indicated from mean survival times along with mortality percentages of hydrocortisone-treated groups (Table 3) that the most virulent tested isolate was *A. ochraceus* fm90 isolated from medical source with mortality percentage up to 50 % and mean survival time up to 2.75 days, followed by *A. oryzae* fm90 from environmental source with mean survival time 11.50 days and mortality percentage 40%. *A. terreus* fm90 from environmental source which caused mean survival time 12 days and mortality percentage 20%, came next. Then finally *A. flavus* fm90 isolated from medical source gave mortality percentage of 0% and mean survival time of 30 days.

TABLE 2. Extracellular enzymes activity of *Aspergillus* species isolated from different sources.

Source of isolation			*Aspergills* species	Esterase activity	1/phospholipase activity	Protease activity
Medical isolates	Patient no.	1 (Tissue of CV)	*A.candidus*	0.031 ^{hijk}	N.d	0.445 ^{b}
		2 (Chest tube)	*A. flavus*	0.020 ^{klmn}	1.194 ^{fg}	0.403 ^{c}
		3 (Tissue of CV)	*A. flavus*	0.021 ^{klmn}	1.275 ^{defg}	0.222 ^{ghi}
		4 (Tissue of CV)	*A. niger*	0.024 ^{Ijklm}	N.d	0.352 ^{de}
		5 (Tissue of CV)	*A. flavus*	0.044 ^{fgh}	1.357 ^{cde}	0.378 ^{cd}
		6 Wound swab	*A. terreus*	0.043 ^{fgh}	N.d	0.065 ^{l}
		7 (Tissue of CV)	*A. fumigatus*	0.009 ^{lmno}	1.428 ^{b}	0.200 ^{Ij}
		8 Sputm	*A. fumigatus*	0.028 ^{Ijkl}	1.757 ^{a}	0.300 ^{f}
		9 Sputm	*A. fumigatus*	0.011 ^{lmn}	1.242 ^{efg}	N.d
		10 CSF	*A. clavatus*	0.047 ^{efg}	1.222 ^{efg}	0.225 ^{ghi}
		11 Sputm	*A. flavus*	0.008 ^{mno}	1.355 ^{cde}	0.351 ^{de}
		12 Nail	*A. niger*	n.d	1.290 ^{def}	N.d
		13 Sputm	*A. clavatus*	0.064 ^{cd}	1.340 ^{defg}	0.195 ^{j}
		14 Sputm	*A. ochraceus*	^{gh}0.148 ^{a}	1.395 ^{cd}	0.550 ^{a}
		15 Sputm	*A. flavus*	0.043 ^{f}	N.d	0.053 ^{lm}
Environmental isolates	Soil	Dar El-Salam	*A. fumigatus*	0.060 ^{cde}	N.d	0.121 ^{k}
			A. flavus	0.001 ^{no}	N.d	N.d
			A. terreus	0.144 ^{a}	N.d	N.d
		Zamalek	*A. niger*	n.d	1.292 ^{def}	0.200 ^{Ij}
			A. flavus	0.070 ^{c}	1.233 ^{defg}	0.252 ^{g}
		Kasr- Eleiny Hospital	*A. ochraceus*	0.036 ^{ghIj}	N.d	N.d
			A. alutaceus	0.031 ^{hIjk}	N.d	N.d
			A. fumigatus	0.053 ^{def}	1.297 ^{def}	N.d
		Manial	*A. niger*	n.d	N.d	0.004 ^{n}
			A. terreus	0.100 ^{b}	1.225 ^{efg}	N.d
			A. nidulans	0.043 ^{ghi}	N.d	N.d
		Cairo University	*A. terreus*	0.018 ^{klmn}	N.d	0.141 ^{k}
			A. niger	n.d	1.453 ^{bc}	N.d
		Giza Zoo	*A. terreus*	0.024 ^{Ijklm}	N.d	N.d
			A. flavus	0.023 ^{jklm}	1.230 ^{efg}	0.013 ^{mn}
			A. niger	n.d	N.d	0.333 ^{ef}
	Outdoor	Microbiology Laboratory in Kasr El-Einy Hospital	*A. clavatus*	n.d	N.d	N.d
			A. restrictus	0.004 ^{no}	N.d	0.120 ^{k}
			A. cervinus	0.03 ^{hIjk}	N.d	0.110 ^{k}
		Office	*A. niger*	0.004	N.d	0.002
			A. restrictus	0.016 ^{lmno}	1.263 ^{defg}	0.140 ^{k}
	Indoor	Air Conditioner Filter	*A. niger*	0.002 ^{no}	N.d	N.d
		Desk	*A. clavatus*	0.004 ^{no}	1.148 ^{g}	o.239 ^{gh}
			A. oryzae	0.068 ^{c}	1.572 ^{b}	0.348 ^{de}
		Wooden floor	*A. oryzae*	0.053 ^{def}	1.284 ^{defg}	0.040 ^{lmn}
LSD (P≤ 0.05)				**0.011**	**0.130**	**0.041**

- N.d: Not detected, CV: Cuspid Valve, CSF: Cerebrospinal fluid.
- Means followed by the same letters are non-significant.

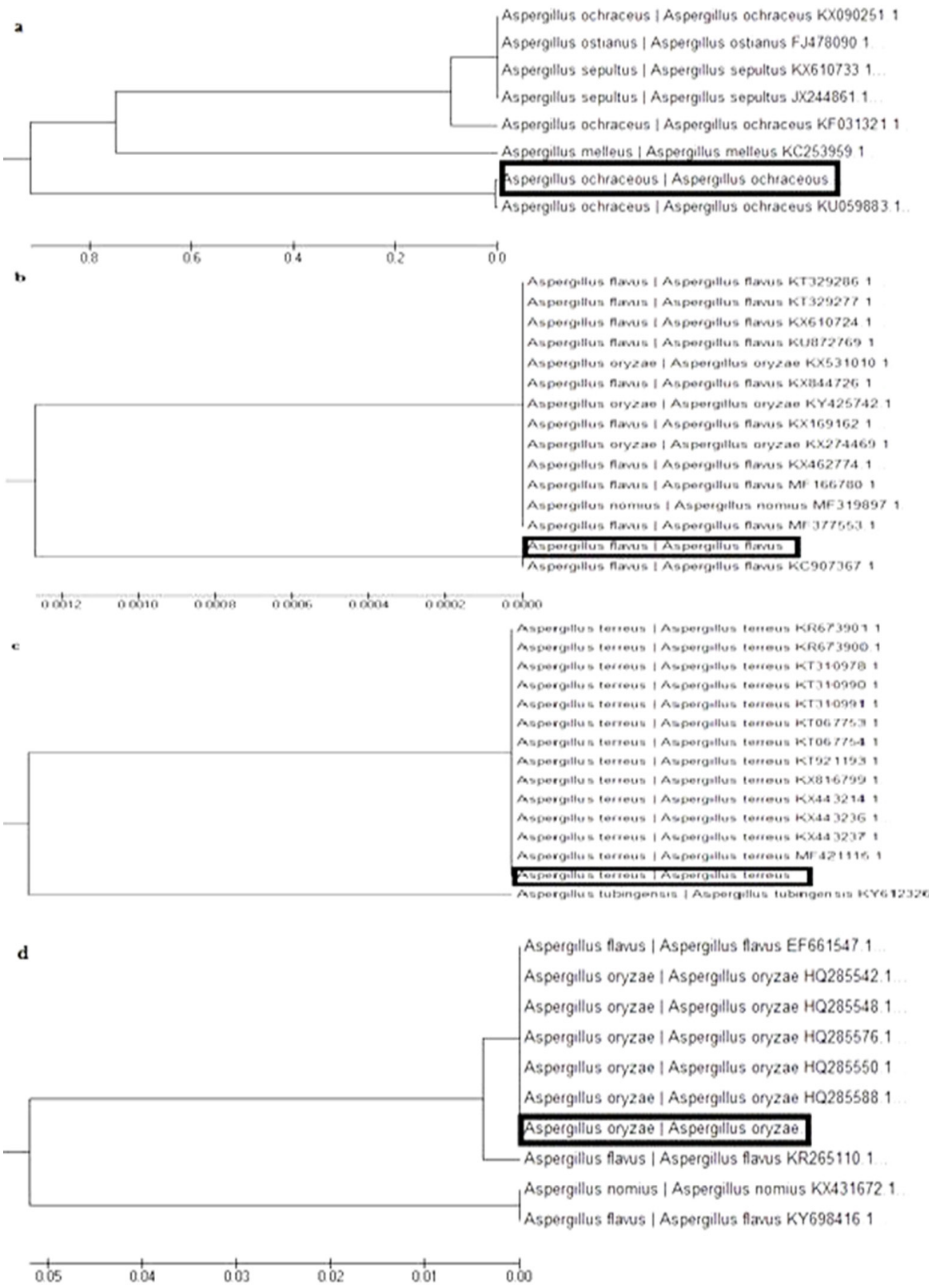

Fig. 1. Phylogenetic tree showing genetic relationship between the outlined isolate; (a) *Aspergillus ochraceous*, (b) *Aspergillus flavs*, (c) *Aspergillus terreus*, (d) *Aspergillus oryzae* and other closely related reference microorganisms.

TABLE 3. Comparison of different *Aspergillus* cultures obtained from various tissues in different mice groups.

Species	Source of isolation	Treatment before infection	Mean survival time (days)	Mortality %	Mean count (CFU)/ corresponding mean survival time		
					Lung	Liver	Kidney
Control		Untreated	30.00	00%	0	0	0
		Hydrocortisone-treated	30.00	00%	0	0	0
A. ochraceus fm90	Medical	Untreated	3	40%	833.3	489.0	1766.6
		Hydrocortisone-treated	2.75	50%	1166.6	10193.32	4083.3
A. oryzae fm90	Environmental	Untreated	30.00	00%	-	-	-
		Hydrocortisone-treated	11.50	40%	715.3	1033.11	125
A. terreus fm90	Environmental	Untreated	13.00	10%	83.3	2976.1	1833.3
		Hydrocortisone-treated	12.00	20%	166.6	13000	3750
A. flavus fm90	Medical	Untreated	30.00	00%	-	-	-
		Hydrocortisone-treated	30.00	00%	-	-	-

Determination of CFU/corresponding mean survival time in different organs

It is worthy to mention that the fungal burden represented as (CFU/corresponding mean survival time) in hydrocortisone-treated mice is greater in liver or kidneys or both than that of untreated mice infected by the same isolate. As an example for mice infected by *A. ochraceus* fm90, CFU/corresponding mean survival time in liver of untreated mice was 489.0 CFU/day while it rose to 10193.32 CFU/day in hydrocortisone-treated mice infected by the same isolate. The same applied for *A. terreus* fm90 as CFU/ corresponding mean survival time in liver and kidneys of untreated mice were 2976.1 and 1833.3 CFU/day, respectively, while it rose to 13000 and 3750 CFU/day, respectively in hydrocortisone-treated mice. This indicates that hydrocortisone treatment deteriorated the host's immunity and thus the infection became more disseminated.

Histopathological examination

In the current work, lung infection of the dead mice was further examined histopathologically (Fig. 2). Infected lungs sections were formalin fixed for histological study. The fixed tissues were embedded in paraffin wax and stained with hematoxylin and eosin (H&E) for light microscopical examination. Thus disease symptoms such as bronchioectasia (permanent enlargement of parts of the airways of the lung), leucocytic cells infiltrations (Infiltration is the diffusion or accumulation in a tissue or cells of foreign substances or in amounts in excess of the normal), congested capilleries and hemorrhages were encountered.

Evaluation of some virulence genes using SYBR Green real-time PCR

Specificity of the primers

From the results reached in the experiment, the most pathogenic *A. ochraceus* fm90 (isolated from a patient) and the least pathogenic *A. flavs* fm90 (isolated from a patient) were chosen for studying the expression levels of protease (alkaline serine protease, PR1), phospholipase (lysophospholipase, Plb1), esterase (lipase/serine esterase) and Asp- haemolysin genes.

Agarose gel analyses of conventional PCR products from *in vitro* cultures are shown in Fig. 3. A single band of the expected size shown in Table 1 (181, 143, & 150bp for esterase (lipase/serine esterase), protease (alkaline serine protease, PR1), and phospholipase (lysophospholipase, Plb1), respectively) for all primer pairs was observed when using cDNA of *A. ochraceus* and *A. flavus* as a template (Fig. 3).

Transcription of protease (alkaline serine protease, PR1), phospholipase (lysophospholipase, Plb1), esterase (lipase/serine esterase) and Asp- haemolysin genes

Protease (alkaline serine protease, PR1), phospholipase (lysophospholipase, Plb1), esterase (lipase/serine esterase) and Asp- haemolysin

genes expression were tested by Real Time PCR (qPCR) in normal growth conditions for *A. ochraceus* fm90 and *A. flavus* fm90 (Table 4 and Fig. 4). Reactions are characterized by the PCR cycle where the target amplification is first detected. This value is usually referred to as cycle threshold (C_t). C_t is the time at which fluorescence intensity is greater than background fluorescence. Consequently, the greater the quantity of target DNA in the starting material, the faster a significant increase in fluorescent signal will appear, yielding a lower C_t. A threshold cycle (C_t) under that of negative control sample indicated a positive result.

For both phospholipase and protease genes, the highest expression level was observed in *Aspergillus ochraceus* fm90 while it was unexpressed in *Aspergillus flavus* fm90 compared to negative control sample according to C_t values (Table 4). Esterase expression was only detected in *Aspergillus ochraceus* fm90 (C_t= 29.93) wich is less than that of negative sample (C_t= 34.83), while no fluorescence was detected for *Aspergillus flavus* fm90.

Weak expression of Asp-hemolysin was detected in *Aspergillus flavus* fm90 *(*C_t= 34.28) while it was unexpressed in *Aspergillus ochraceous* (C_t= 36.55) which exceeds C_t value of negative control sample (C_t= 34.83).

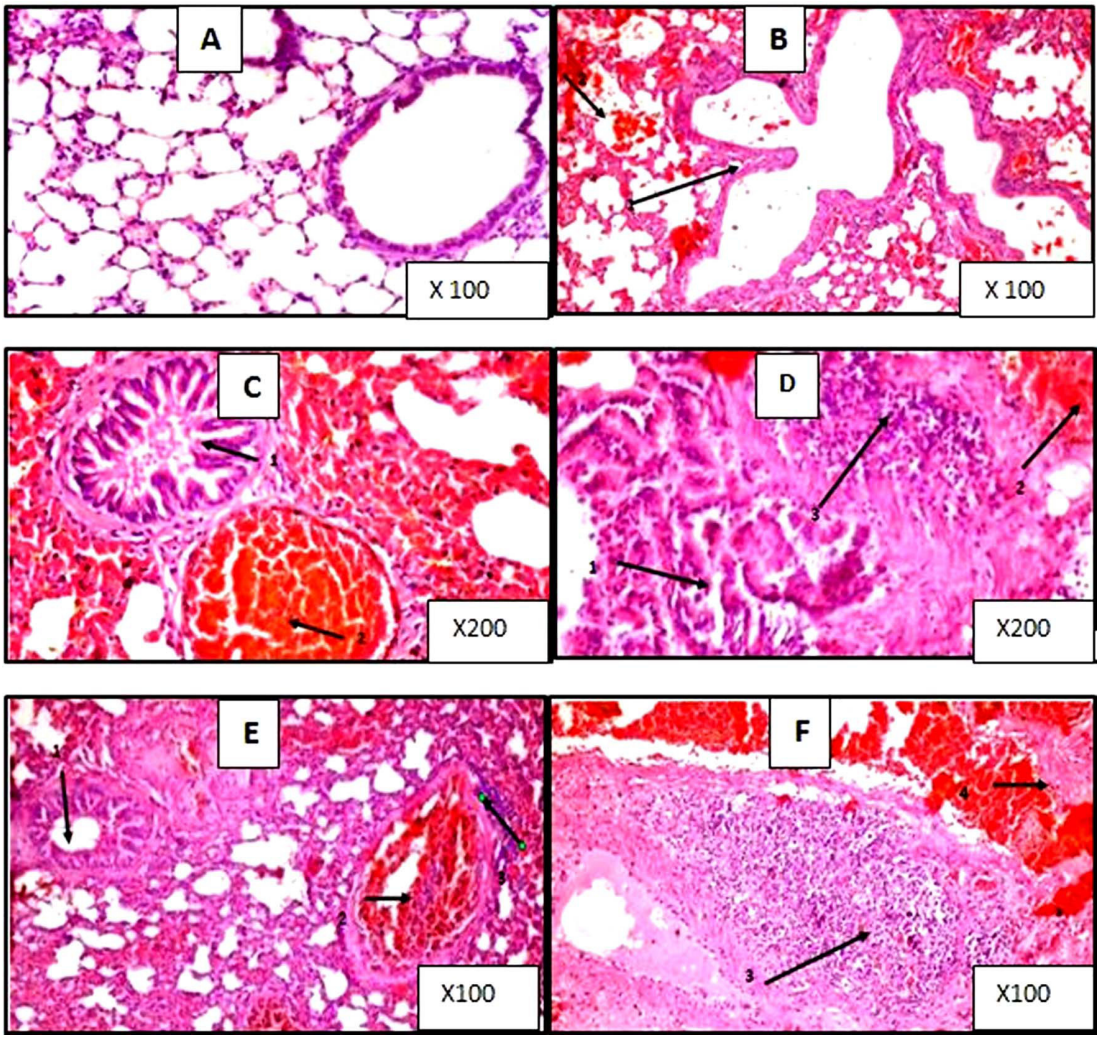

Fig. 2. Histopathological examination of mice lungs sections stained by hematoxylin & eosin dye showing: 1- Hyperplasia of the bronchiole, 2- Conjestion of blood vessels, 3- Leucocytic cells infiltration and 4- Hemorrhage [(A) Non infected, (B) Untreated mice infected by *Aspergillus ochraceus*, (C) Hydrocortisone-treated mice infected by *Aspergillus ochraceus*, (D) Hydrocortisone-treated mice infected by *Aspergillus oryzae*, (E) Untreated mice infected by *Aspergillus terreus* and (F) Hydrocortisone-treated mice infected by *Aspergillus terreus*].

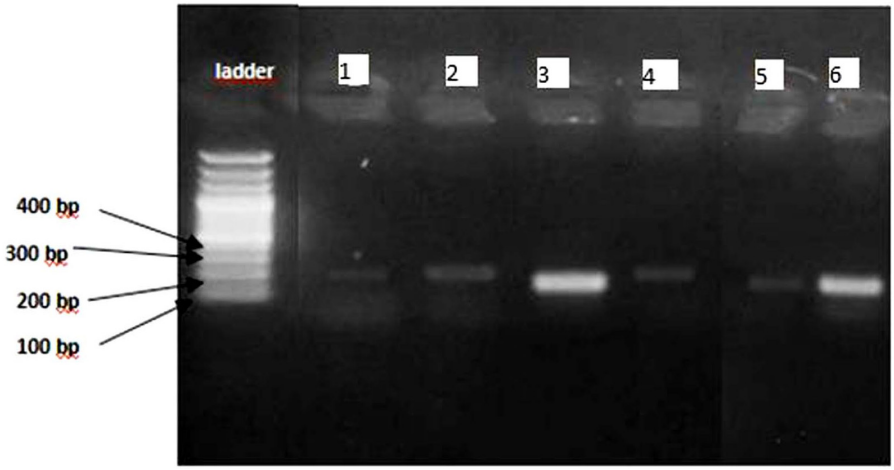

Fig. 3. Conventional PCR products separated on a 1.5% agarose gel for all primer pairs. Lanes 1 & 2: Alkaline serine protease gene from *A. flavus* fm90 and *A. ochraceus* fm90, respectively, lanes 3 & 4: Phosphplipase plb1 gene from *A. ochraceus* fm90 and *A. flavus* fm90, respectively and lanes 5 & 6: Lipase/serine esterase gene from *A. flavus* fm90 and *A. ochraceus* fm90, respectively.

TABLE 4. Expression of each gene expressed as C_t value (The cycle at which fluorescence from amplification exceeds the background fluorescence).

Gene	Threshold cycle (C_t)		
	Negative control	*A. flavus* fm90	*A. ochraceous* fm90
Protease	32.18	34.15	28.14
Phosphpolipase		33.99	26.8
Esterase	34.83	N.d	29.93

N.d: Not detected.

Discussion

Aspergillosis is considered the most important opportunistic disease for immunocompromised patients (Garcia-Vidal et al., 2008).

Enzyme production is a factor that participates in the virulence process, which plays an important role in the pathogenicity of pathogenic organisms (Schaller et al., 2005).

In the current study, highest phospholipase and Esterase occurrence were achieved by *Aspergillus* species isolated from patients with Aspergillosis. 12 out of 15 (80%) and 14 out of 15 (93.3%), respectively. Protease was positive in 86.7% (13/15 isolates) of Aspergilli isolated from patients with Aspergillosis, while was positive only in 43.8% (7/16 isolates) of Aspergilli isolated from soil and was detected in 77.8% (7/9) in isolates from air samples (outdoor and indoor).

In this respect, Birch et al. (2004) compared environmental and clinical isolates of *A. fumigatus* and variations in production were observed, where clinical isolates were more significant producers of phospholipase C than environmental isolates.

In close relation with this study, Raksha & Urhekar (2017) tested proteinolytic activity of *Aspergillus* isolates (total 750 samples, were included, 350 and 400 samples from patients and environment, respectively). Proteinolytic activity was seen in 87.17% of patient's samples as compared to 41.02% of environmental samples, the authors also studied virulence factors (α–amylase, lipase, proteinase, pectinase, phospholipase and haemolysin) in both patients and environment *Aspergillus* isolates. However, number of positivity in environmental samples is less than those of patient samples. It appeared that virulence factors are inherently present in environment samples and active in small percentage for survival in environment. But on entry in human tissues, their activity is increased to resist the unfavorable condition, defensive mechanism of human bodies which tries to destroy the microorganisms. Hence, for survival in human tissues, the virulence factors activity is geared up to oppose human protective mechanisms.

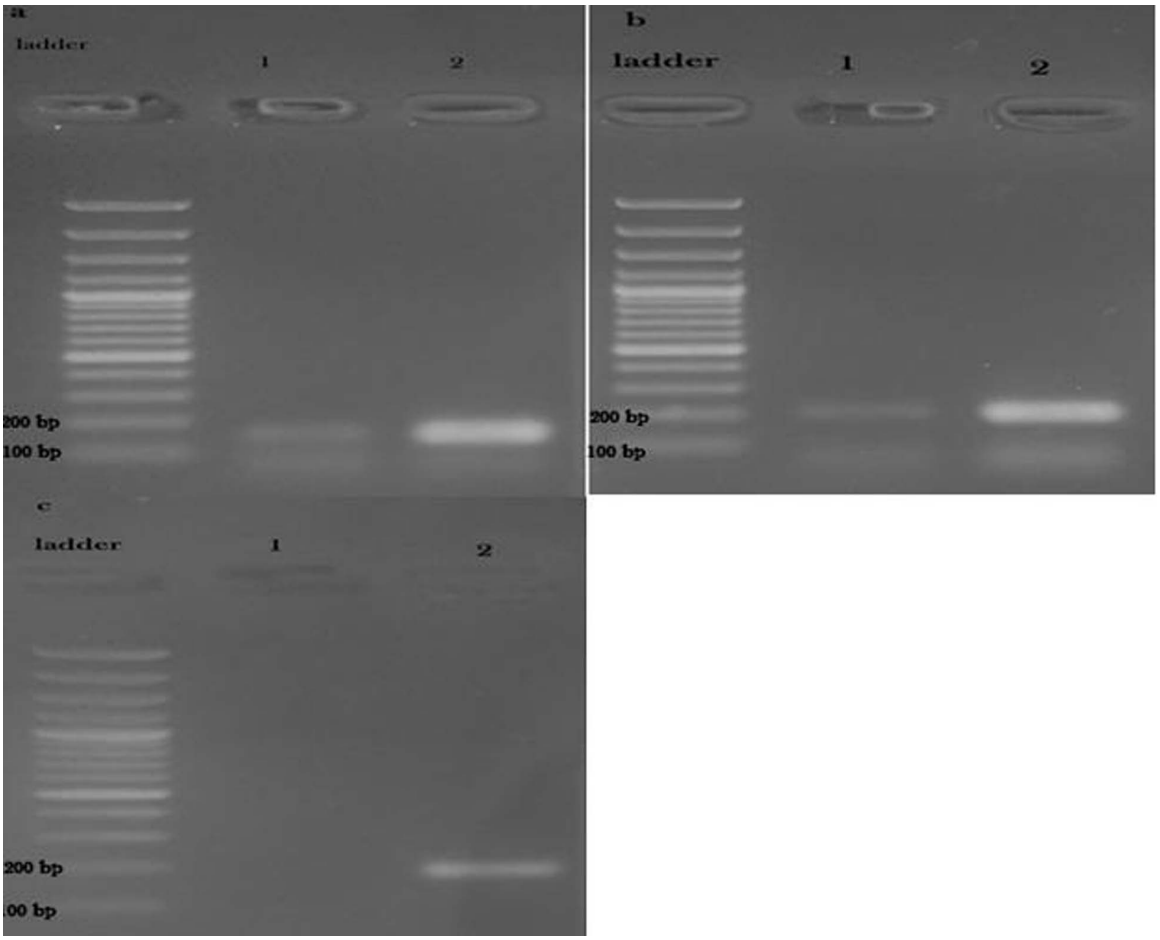

Fig. 4. Expression of phospholipase (A), protease (B), esterase (C), from (1) *A. flavus* fm90 and (2) *A. ochraceus* fm90 cultures.

The current study considered analyzing the relation between some enzymatic activities (phospholipase, esterase, protease) of the isolated *Aspergillus* species and their potential in causing Aspergillosis in mice. Also, comparing results of isolates from environmental sources and from patients causing aspergillosis was examined. Accordingly, four *Aspergillus* isolates with highest and lowest enzymatic activities were chosen from both environmental and patient sources. It was found that *Aspregillus oryzae* isolated from indoor environment along with *Aspergillus ochraceus* isolated from patient no. 14 achieved the highest enzymatic activities while both *Aspergillus terreus* isolated from Cairo University soil and *Aspergillus flavus* isolated from patient no. 15 were the least enzymatically active isolates. So those isolates were first selected and identified at the molecular level to test their pathogenicity.

To study the relation between the enzymatic activity of an isolate and its pathogenicity, the selected isolates mentioned before were injected in mice and observed for 4 weeks to determine percentage of mortality and mean survival time. Only dead mice were dissected and fungal counts in lung (left), kidneys and liver were determined along with histopathological examination of lungs.

It was indicated from mean survival times along with mortality percentages of hydrocortisone-treated groups that the most virulent tested isolate was *A. ochraceus* fm90 isolated from medical source with mortality percentage up to 50% and mean survival time up to 2.75 days, followed by *A. oryzae* fm90 from environmental source with mean survival time 11.50 days and mortality percentage 40%. *A. terreus* fm90 from environmental source which caused mean survival time 12 days and mortality percentage 20%, came next. Then finally *A. flavus* fm90 isolated from medical source gave mortality percentage of 0% and mean survival time of 30 days.

In the present study, it was found that fungal burden in lung tissue (Table 3) is inversely proportional to mean survival time of the mice. In general, it was detected that fungal burden in kidney tissue is inversely proportional to that of liver.

In agreement with this study, the results of Anand et al. (2015) revealed that the cortisone administration enhanced the mice susceptibility to the *Aspergillus* spp spores. Systemic spread following lung infection or direct extension from paranasal sinuses could cause invasive aspergillosis in such organs as kidneys, brain etc.

Similarly, Mirkov et al. (2014) performed histological lung tissue examination after 1 and 7 days of infection. The results showed inflammatory responses characterized by interstitial widening and perivascular leukocyte infiltration at both time points following infection.

From the present study, *A. ochraceus* fm90 (from a medical source) and *A. oryzae* fm90 (from an environmental source) were the highest chosen enzymatically active isolates and they both were more pathogenic than *A. flavus* fm90 and *A. terreus* fm90 which were the least enzymatically active isolates from medical and environmental sources, respectively. It can therefore be deduced that there was a direct relation between enzymatic activities of an *Aspergillus* isolate and its virulence regardless of its source of isolation (medical or environmental). Therefore, enzymes can be considered as virulence factors.

It was thought advisable to study the gene expression levels of the tested enzymes in this study of the highest (*A. ochraceus* fm90) and lowest (*A. flavus* fm90) pathogenic isolates from the mice infection experiment in this work.

As indicated by C_t values (Table 4) along with gel electrophoresis of the real-time PCR products (Fig. 4), it was found that expression levels for each of protease (alkaline serine protease, PR1), phospholipase (lysophospholipase, Plb1) and esterase (lipase/serine esterase) genes were expressed with a higher level in the most pathogenic tested isolate (*A. ochraceus* fm90) than in the least pathogenic one (*A. flavus* fm 90 MF284670). These results were in accordance with the physiological tests for enzymatic activities which suggest the implication of those genes in the virulence of this isolate. For the single isolate *A. ochraceus* fm90, it was found that the highest expression level was achieved in phospholipase gene which means that phospholipase is implicated in virulence of this isolate to a greater extent than other enzymes, followed by protease and then esterase. These observations are in accordance with the tested enzymatic activities and pathogenicity experiment. Figure 4 depicts the PCR products of genes protease (alkaline serine protease, PR1), phospholipase (lysophospholipase, Plb1) and esterase (lipase/serine esterase) for *Aspergillus ochraceus* fm90 and *Aspergillus flavus* fm90. The expected sizes of real time PCR products were 143, 150, 181 bp for protease (alkaline serine protease, PR1), phospholipase (lysophospholipase, Plb1) and esterase (lipase/serine esterase), respectively (Table 1) for both *Aspergillus ochraceus* fm90 and *Aspergillus flavus* fm90. The resulted sizes (Fig. 4) were approximately similar to the expected sizes. However, esterase (lipase/serine esterase) was only detected in case of *Aspergillus ochraceus* fm90.

In this respect, Zhang et al. (2005) stated that in *A. fumigatus*, various virulence factors have been recorded including, pksP, fos-1, rhbA, cpcA, lysF, and pabA each of which is required for murine IA (invasive aspergillosis). If a gene is expressed during infection at higher levels compared to *in vitro* cultured fungus, this observation may mean that the gene plays a role in virulence, this was proven by some studies.

Conclusion

A close relation can be deduced between enzymatic activity and pathogenicity of the tested isolates *in vitro*. Based on the results of biochemical assay of enzymatic activities of the selected *Aspergillus* species along with the *in vivo* pathogenicity experiment and finally evaluation of genes expression level by real-time PCR, it appears that phospholipase and protease can be considered as potent virulence factors used by the most pathogenic tested *Aspergillus* isolate (*A. ochraceus* fm90) as they were expressed to a higher extent than in *A. flavus* fm90. It can also be concluded that both medical and environmental *Apergillus* isolates can be pathogenic, so pathogenicity is not related to the source of isolation and it is rather related to its physiological (enzymatic) activity. And this in

turn considered a risk calling for attention along with public awareness as humans are exposed daily for considerable amount of fungal spores from environmental habitats.

To combat invasive aspergillosis (IA), some protective procedures should be followed. These include constructing periodic survey studies of invasive aspergillosis local epidemiology. Regular checks should be considered for the fungal burden in hospitals. The Centers for Disease Control and Prevention guidelines recommended high-efficiency particulate air (HEPA) filter systems for immunocompromised patients. Such high risk patients should wear HEPA masks when exposed to dust as recommended by Centers for Disease Control and Prevention (CDC, 2007).

References

Anand, R., Shankar, J., Tiwary, B.N. and Singh, A.P. (2015) *Aspergillus flavus* induces granulomatous cerebral aspergillosis in mice with display of distinct cytokine profile. *Cytokine,* **72**(2), 166-72.

Birch, M., Denning, D.W. and Robson, G.D. (2004) Comparison of extracellular phospholipase activities in clinical and environmental *Aspergillus fumigatus* isolates. *Sabouraud,* **42**(1), 81-6.

Centers for Disease Control and Prevention (CDC) (2007) Guideline for Isolation Precautions: Preventing Transmission of Infectious Agents in Healthcare Settings.

Chhabra, D. and Dhakad, N.K. (2008) Study on pathogenicity of the *Aspergillus* species in experimentally immunosuppressed mice. *Vet. World,* **1**(3), 69-70.

Coral, G., Arikan, B., Unaldi, M.N. and Guvenmez, H. (2003) Thermostable alkaline protease produced by an *Aspergillus niger* strain. *Annals of Microbiol.* **53**(4), 491-8.

Garcia-Vidal, C., Upton, A., Kirby, K.A. and Marr, K.A. (2008) Epidemiology of invasive mold infections in allogeneic stem cell transplant recipients: Biological risk factors for infection according to time after transplantation. *Clin. Infect. Dis,* **47**(8), 1041-50.

Hoekstra, E.S., Samson, R.A. and Summerbell, R.C. (2000) Methods for the detection and isolation of fungi in the indoor environments. In: "*Introduction to Food and Airborne Fungi*", pp. 298-305.

Johnson, L.F., Curi, E.A., Bond, J.H. and Fribourg, H.A. (1960) "*Methods for Studying Soil Microflora-Plant Disease Relationship*". *B*urgess Publishing, Minneapolis, U.S.A, 178p.

Kauffman, C.A., Pappas, P.G., Sobel, J.D. and Dismukes, W.E. (2011) "*Essentials of Clinical Mycology*", pp. 321-335, Springer, New York.

Kendall, P.A. (1963) Use of the ninhydrin reaction for quantitative estimation of amino groups in insoluble specimens. *Nature,* **197**(4874), 1305-6.

Khosravi, A.R., Riazipour, M., Shokri, H. and Mousavi, M. (2008) Intracellular esterase activity of *Candida albicans* and its correlation with pathogenicity in mice. *Journal de Mycol. Méd/Jour. of Med. Myc.* **18**(3), 134-40.

Khosravi, A.R., Omran, S.M., Shokri, H., Lotfi, A. and Moosavi, Z. (2012) Importance of elastase production in development of invasive aspergillosis. *Jour. de Mycol. Méd/Jour. of Med. Mycol.* **22**(2), 167-72.

Kothary, M.H., Chase, T. and Macmillan, J.D. (1984) Correlation of elastase production by some strains of *Aspergillus fumigatus* with ability to cause pulmonary invasive aspergillosis in mice. *Infect. Immun*. **43**(1), 320-5.

Latgé, J.P. (2001) The pathobiology of *Aspergillus fumigatus*. *Trends in Microbiology*, **9**(8), 382-389.

Lovrien, R.E., Gusek, T. and Hart, B. (1985) Cellulase and protease specific activities of commercially available cellulase preparations. *J. Appl. Biochem*. **7**(4-5), 258-72.

Mirkov, I., El-Muzghi, A.A., Djokic, J., Ninkov, M. and Aleksandrov, A.P. (2014) Pulmonary immune responses to *Aspergillus fumigatus* in rats. *Biomed. Envir. Sci.* **27**(9), 684-94.

Patterson, T.F., Kirkpatrick, W.R., White, M., Hiemenz, J.W., Wingard, J.R., Dupont, B., Rinaldi, M.G., Stevens, D.A. and Graybill, J.R. (2000) Invasive aspergillosis. Disease spectrum, treatment practices, and Outcomes. I3 Aspergillus Study Group. *Medicine,* **79**(4), 250-260.

Price, M.F., Wilkinson, I.D. and Gentry, L.O. (1982) Plate method for detection of phospholipase activity in *Candida albicans*. *Sabouraud,* **20**(1), 7-14.

Raksha, G.S. and Urhekar, A.D. (2017) Virulence factors detection in *Aspergillus* isolates from clinical and environmental samples. *Journal of Clin. Diag. Res. (JCDR),* **11**(7), DC13.

Raper, K.B. and Fennell, D.I. (1965) "*The Genus Aspergillus*". Williams, Baltimore, U.S.A.

Rementeria, A., López-Molina, N., Ludwig, A., Vivanco, A.B., Bikandi, J. and Pontón, J. (2005) Genes and molecules involved in *Aspergillus fumigatus* virulence. *Rev. Iberoam. Micol.* **22**(1), 1-23.

Schaller, M., Borelli, C., Korting, H.C. and Hube, B. (2005) Hydrolytic enzymes as virulence factors of *Candida albicans*. *Mycoses,* **48**(6), 365-77.

Walsh, T.J., Anaissie, E.J., Denning, D.W., Herbrecht, R., Kontoyiannis, D.P., Marr, K.A. and Van Burik, J.A. (2008) Treatment of aspergillosis: Clinical practice guidelines of the infectious diseases society of America. *Clinical Infectious Diseases,* **46**(3), 327-360.

Xess, I., Mohanty, S., Jain, N. and Banerjee, U. (2004) Prevalence of *Aspergillus* species in clinical samples isolated in an Indian tertiary care hospital. *Ind. J. Med. Sci.* **58**(12), 513.

Zhang, L., Wang, M., Li, R. and Calderone, R. (2005) Expression of *Aspergillus fumigatus* virulence-related genes detected *in vitro* and *in vivo* with competitive RT-PCR. *Mycopathologia,* **160**(3), 201-6.

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انزيمات التحلل المائى كعوامل ضراوة محتملة لفطرة اسبرجللس اوكريشيس فى مرض الاسبرجللوزس

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كان المفهوم الرئيسي لهذه الدراسة هو تقييم إمكانية أنزيمات التحلل المائي كعوامل ضراوة لأنواع فطرة الاسبرجللس خلال عملية عدوى الاسبرجللوزس. تم عزل أربعين نوعًا من أنواع الاسبرجلس منها 15 عزلة طبية وكذلك عزلات بيئية (25 عزلة) من التربة والبيئات الخارجية والداخلية. تم قياس انشطة البروتياز، فسفوليباز و الأستريز. تم تقييم القدرة على احداث المرض في بعض أنواع الاسبرجلس في الجسم الحي. تمثل ذلك في أوقات البقاء على قيد الحياة ، ونسب الوفيات، والأعداد الفطرية في أعضاء مختلفة، والفحص الهستوباثولوجي لأنسجة الرئة. تمت دراسة مستويات التعبير عن جينات البروتياز، فسفوليباز وإستريز بواسطة PCR real time. تم اختيار أربع عزلات ذات تأثير معنوى من الناحية الفسيولوجية من أصل أربعين عزلة وتم تعريفها حتى المستوى الجزيئي. سجلت العزلة fm90 *Aspergillus ochraceus* أعلى نسبة مرضية ممثلة في النسب المئوية للوفيات وأوقات البقاء على قيد الحياة لدى الفئران، بينما كانت العزلة fm90 *A. flavus* هى الأقل مسببة للإمراض. كانت معدلات التعبير عن جينات البروتياز، فسفوليباز وإستريز أكبر في fm90 *A. ochraceus* عنها في fm90 *A. flavus*. يمكن أن نستنتج أن الإمراض ربما يكون مرتبطا بالأنشطة الفسيولوجية (الأنزيمية) للعزلات. أيضا، يمكن للتنوع في مستويات التعبير عن جينات البروتياز و فسفوليباز في fm90 *A. ochraceus* و fm90 *A. flavus* أن يدل على تورطهم المحتمل في عملية الإمراض.