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Study of Hemolysin Gene "*aspHS*" and Its Phenotype in *Aspergillus Fumigatus*

Majid Zarrin[1, 2*], Farzaneh Ganj[2]

[1]Infectious and Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran; [2]Department of Medical Mycology, Medical School, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

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***Correspondence:** Majid Zarrin. Infectious and Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran; Department of Medical Mycology, Medical School, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. E-mail: mjzarrin@yahoo.co.uk

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Abstract

AIM: The main goal of this study was to analysis the "*aspHS*" gene and its phenotype in *A. fumigatus.*

METHODS: Fifty-three *A. fumigatus* strains, including environmental, clinical and reference isolates, were used in this research. PCR was carried out based on Asp-hemolysin gene sequence. Two restriction enzymes *TagI* and *NcoI* were employed for digestion of PCR products.

RESULTS: PCR products of 180 and 450 bp were generated for all *A. fumigatus* isolates. Digestion of the *aspHS* gene 180 bp amplicons with *TagI* and 450 *bp amplicons with TagI and NcoI produced the expected bands for most isolates. Hemolysin production of A*. fumigatus isolates was evaluated on sheep blood agar (SBA).

CONCLUSION: In conclusion, our results provide evidence hemolysin activity and analysis of *aspHS* gene of *A. fumigatus*. These data may be useful in early diagnosis of *A. fumigatus* infections.

Introduction

An aspergillosis is a group of infections because of opportunistic infection caused by different species of *Aspergillus*. Among this disease, invasive aspergillosis (IA) is a severe nosocomial infection winch usually has a high mortality rate. *Aspergillus fumigatus* is most common etiological cause of IA, followed by *A. flavus*, *A. niger*, *A. terreus* [1], [2]. Asp-hemolysin is a hemolytic and cytolytic toxin from *A. fumigatus* [3].

Asp-hemolysin gene has been cloned, and sequence of the gene reported [4]. The primary sequencing of Asp-hemolysin gene product was predicted from cDNA. It has 131 amino acid residues and a molecular mass of 14 275.

The hemolysin has negatively charged domains. It enables the *A. fumigatus* to disrupt blood cells and can be identified in infected patients. The produced hemolysin by *A. fumigatus* promotes infection with *Aspergillus* species and also other opportunistic infections [5], [6], [7].

A. fumigatus possess a special combination of dissimilar virulence-related factors, creation it the most important global filamentous fungi pathogen. Nevertheless, although the hemolysin has toxicity effects, it appears not to be a major virulence factor but a compound which can increase the effects of other toxic pathogenicity factors [5], [6]. Hemolysin is lethal to chickens and mice, and it also is lytic for erythrocytes of humans, sheep and rabbits. This toxin

has cytotoxic effects on macrophages and endothelial cells in vitro [8], [9].

Produced toxins seem with the fungus to defend itself from killers and competitors in environment. Moreover, these toxins could contribute to pathogenesis *A. fumigatus* because they are able directly invade the host tissue [10], [11].

The main goal of the current study was to compare hemolysin phenotype and genotype features among a variety of *A. fumigatus* isolates.

Material and Methods

Isolates of A. fumigatus

Fifty-three *A. fumigatus* isolates were used in this study. Four reference strains, including *A. fumigatus* IBRC-M 30033, IBRC-M 30040, IBRC-M 30048 and PTCC 5009, and 10 clinical and 39 environmental isolates of *A. fumigatus* were included. Eight clinical isolates were kindly provided by Dr Mojtaba Taghizadeh (Mazandaran University of Medical Sciences, Mazandaran, Iran). The environmental isolates were obtained from soil or air samples collected in Ahvaz, Iran.

The isolates were incubated on Sabouraud dextrose agar (Merck KGaA, Darmstadt, Germany) at 37°C. *A. fumigatus* isolates were identified morphologically. The isolates subcultured three times to obtain a pure culture and stained with lactophenol aniline blue. Vesicles, conidiophores, phialides and conidial arrangement were searched with a light microscope for morphological identification.

Hemolysin production of A. fumigatus isolates

Two microliters of spore suspension 5 × 10^6 from each *A. fumigatus* was inoculated on sheep blood agar (SBA). The Petri dishes were incubated at 37°C for 3 days, triplicate for each isolate. The presence of clear hemolysis in the medium indicated the evidence of hemolysin and recorded as a positive [12].

DNA extraction

One ml thick spore suspension from each *A. fumigatus* isolate was inoculated to an Erlenmeyer flask containing 100 ml yeast extract peptone dextrose medium (Merck KGaA) and incubated in an incubator shaker at 200 rpm under agitation for 48 h at 37°C for mycelia growth. The mycelia were harvested with filters, washed with 0.5 M EDTA and sterile dH2O and ground into a fine powder using liquid nitrogen with a pestle and mortar. One hundred mg powdered mycelium was transferred into a 1.5 ml sterile microtube containing 400 µl lysis buffer (100 mM Tris-HCl, pH 8.0, 30 mM EDTA, pH 8.0, 5% SDS w/v). After the incubation of the microtubes at 100°C for 20 min, 150 µl of 3 M acetate potassium was added. This suspension was kept at -20°C for 10 min and spun at 14,000 x g and 4°C for 10 min. After transferring of the supernatant to a 1.5 ml Eppendorf tube, 250 µl phenol-chloroform-isoamyl alcohol (25:24:1, v / v) was added, and the solution was shortly was vortexed and centrifuged for 10 min at 14,000 x g. The upper solution was transferred to a 1.5 ml tube, and 250 µl of chloroform-isoamyl alcohol (24:1) was added. The microtubes were then briefly vortexed and spun at 4°C and 14,000 x g for 10 min. The upper aqueous was transferred to a new microtube, and an equal volume of ice-cold 2-propanols was added. The solution was maintained at -20°C for 10 min and centrifuged at 14,000 x g for 10 min.

The supernatant was removed, and the pellet was washed with 300 µl ethanol 70%. After removal of the ethanol, DNA pellet was air-dried and dissolved in 50 µl dH2O.

PCR amplification

The fragments of the Asp-hemolysin gene were amplified by using primer sets: F-Asphs (5'-TGGTACAAGGACGGTGACAA-3') and R-Asphs (5'-GTCCCAGTGGACTCTTCCAA-3') for amplification of an 180 bp DNA [13] and Afhem1 (5' –GCATCGGTCCAAGCTTACGCA -3') and Afhm2 (5'—TTAACAGTTGCCAATGGCACC-3") for amplification of an ~450 bp DNA [14]. Set up the PCR reactions for desired fragments to a final volume of 50 µl, containing reaction buffer, 2.2 mM MgCl2, 200 µM of each dNTP, 2.5 unit of Taq DNA polymerase (CinnaGen, Karaj, Iran), a 25 ng DNA template and 50 pmol of each primer.

Amplification conditions used were: For 180 bp fragment; Initial denaturation at 95°C for 10 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 2 min and extension at 72°C for 1 min. For 450 bp fragment; 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1 min, with a final extension at 72°C for 7 min [14]. Amplicons were analysed by 1% agarose gel electrophoresis in a Tris base, acetic acid and EDTA (TAE) buffer, and stained with ethidium bromide.

RFLP analysis

Restriction enzyme pattern of the 180 bp and 450 bp sequences were predicted for restriction endonucleases with Restriction Mapper Version3 software. *Tag*I (Fermentas; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for analysis of 180 bp

fragment was used. *Nco*I (Fermentas; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and *Tag*I were performed for 450 bp fragment of Asp-hemolysin.

The reaction for each restriction enzyme was carried out in a total volume of 20 µl containing 10 units of the enzyme, 2 µl of the related buffer, 5 µl of the PCR product and Ultrapure water (CinnaGen, Karaj, Iran) to generate the 20-µl volume. Digested PCR products were subjected to electrophoresis on a 1.8% agarose gel in TAE buffer, and stained with ethidium bromide.

Sequencing

Several amplicons for each fragment were submitted for direct sequencing (Bioneer Corporation, Daejeon, South Korea). The sequences were searched for in the NCBI database (http://www.ncbi.nlm.nih.gov/). The sequences had 99-100% identity with *A. fumigatus* Asp-hemolysin gene deposited in the NCBI database. The package MEGA5 software (http://www.megasoftware.net) was applied for alignment of sequences.

Results

Hemolysin production test for A. fumigatus isolates

A total of 53 *A. fumigatus* isolates were screened for hemolysin production. The screening was performed with SBA. All 53 *A. fumigatus* isolates (100%) had able to produce hemolysin (Figure 1).

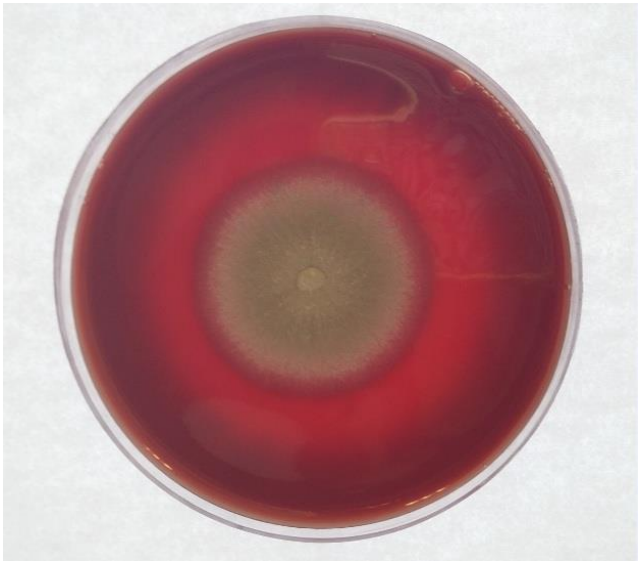

Figure 1: Hemolysin production of A. fumigatus on sheep blood agar (SBA) after 3 days at 37°C

A. fumigatus isolates gave the clear zone in a different ratio. The zone of the hemolysin production of isolates was ranged between 6-7.6 mm in diameter (Table 1).

Table 1: Ability of A. fumigatus isolates in hemolysin production on sheep blood agar at 37°C for 3 days

Sample type	6-6.5 mm		6.6-7 mm		7.1-7.6 mm		Total	
	No.	%	No.	%	No.	%	No.	%
Reference	0	0	1	25	3	75	4	100
Clinical	4	40	2	20	4	40	10	100
Environmental	3	8	24	61	12	31	39	100
Total	7	13	27	51	19	36	53	100

Thirty-six per cent of isolates has produced the zone of 7.1-7.6 mm. From this, the highest activity was belonging to reference isolates with 75% followed by clinical and environmental isolates with 40% and 30% subsequently.

Molecular variation analysis of 180 bp of aspHS gene

Using primers F-Asphs and R-Asphs, a 180 bp was amplified for all tested *A. fumigatus* isolates (Figure 2).

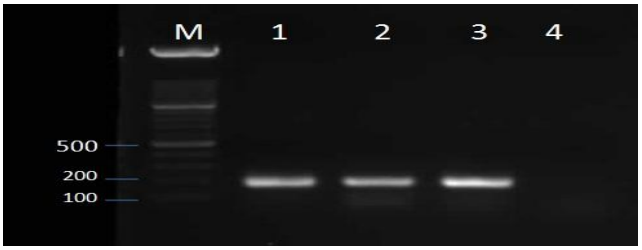

Figure 2: Agarose gel electrophoresis of 180 bp aspHS gene products of the A. fumigatus species with primers F-Asphs and R-Asphs. M, 100 bp ladder; lane 1, IBRC-M30033; lane2, IBRC-30040; Lane3, IBRC-30048; Lane4, no template control

Digestion of the *aspHS* gene amplicons with *Tag*I produced the two expected 115 and 65 bp fragments for 51 isolates. The isolates E1 and E2 showed different pattern after digestion with *Tag*I (Figure 3).

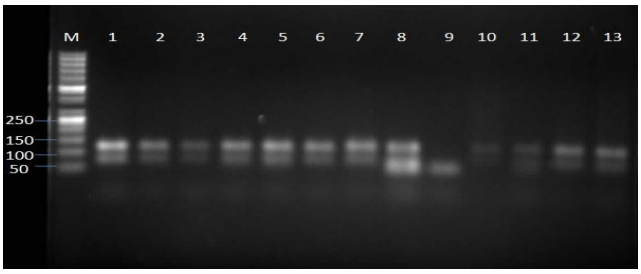

Figure 3: Restriction fragment pattern of 180 bp A. fumigatus aspHS gene digested with TagI. Lane M, 50 bp ladder; lane 1, E25; lane 2, E26; lane 3,E27; lane 4, E31; lane5, E35; lane 6, E37; lane 7, E45; lane 8,E1; lane 9, E2; lane 10, E3; lane11,E4; lane 12, E5; lane 13, E6

Molecular variation analysis of 450 bp of aspHS gene

PCR amplification of the *aspHS* gene with primers AFhem1 and AFhem2 resulted in a 450-bp band for all 53 *A. fumigatus* isolates (Figure 4).

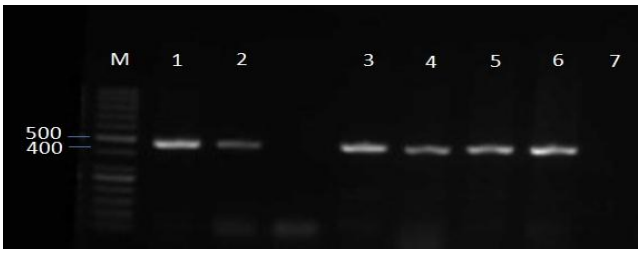

Figure 4: Agarose gel electrophoresis of 450 bp aspHS gene products of the A. fumigatus species with primers AFhem1 and AFhem2. Lane M, 50bp ladder; lane 1, PTCC5009; lane 2, IBRC-M30048; lane 3,E5; Lane 4, E9; lane 5, E10; lane 6, E11; lane 7, Negative control

Digestion of the *aspHS* gene products with *Tag*I produced the 3 expected 50, 110 and 290 bp fragments for all 53 isolates. The isolates E1 and E2 showed a different pattern after digestion with *Tag*I (Figure 5).

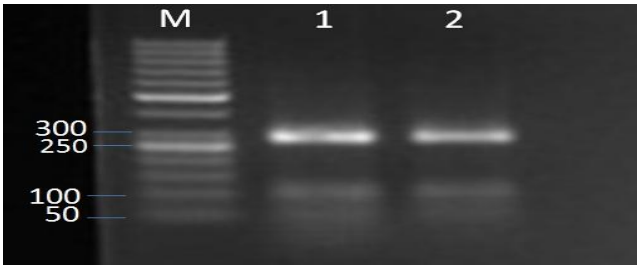

Figure 5: Restriction fragment pattern of 450 bp A. fumigatus aspHS gene digested with TagI. Lane M, 50 bp ladder; lane 1, IBRC-M30033; Lane 2, IBRC-M30040

Digestion with *NcoI* produced the 2 expected 100, 350 bp bands for 52 isolates. The isolates E2 showed different pattern after digestion with *NcoI* (Figure 6).

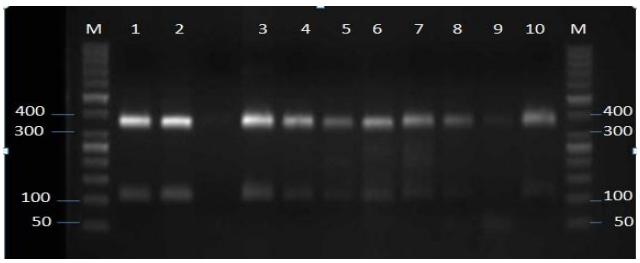

Figure 6: Restriction fragment pattern of 450 bp A. fumigatus aspHS gene digested with NcoI. Lane M, 50bp ladder; lane 1, E25; lane 2, E26; lane 3,E27; lane 4, E31; lane5, E35; lane 6m E37; lane 7, E45; lane 8, E1; lane 9:,E2; lane 10:E3

Sequencing

The PCR products several isolates were sequenced and aligned with references in the NCBI database. The sequences had 100% similarity with *A. fumigatus aspHS* gene sequences deposited in the NCBI database.

Discussion

An important reason for the high mortality connected with IA is its difficulty for early diagnosis. Asp-hemolysin is produced by *A. fumigatus*. It is a hemolytic and cytolytic toxin.The Asp-hemolysin gene is more greatly expressed *in vivo* compared to *in vitro* [15], [16] and also it has lately been described as a main *in vitro* -secreted protein [17]. Asp-hemolysin molecule has hemolytic activity on erythrocytes of rabbit and sheep and also causing in vitro cytotoxic effects on endothelial cells and macrophages in vitro. This molecule can be distinguished during infection in vivo [18].

A. fumigatus is the most important etiological agent of IA. Therefore early recognition of this species is very vital for at-risk patients.

Hemolysin created from A. fumigatus isolates from various sources, clinical and environmental.

One recent research the levels of expression of certain genes such as *gliP*, *aspHS*, *asp f 1*, and *dmaW* were found out by real-time RT-PCR analysis and higher expression was detected in vivo comparing to in vitro [15]. Thes results from these researches suggest overexpression of Asp-hemolysin during infection.

Hemolysin cytotoxicity possibly because of the capability of the hemolysin to inducing the DNA damage and creating mutations in an animal model and cell cultures. Different hemolysin can induce genotoxicity of dietary carcinogens *in vitro* considering that the level of induction was powerfully dependent to species [19], [20].

In our study, all *A. fumigatus* isolates exhibited hemolytic activity. We demonstrated that the hemolytic activity of *A. fumigatus* was significantly higher in clinical isolates compared to environmental isolates in sheep blood SDA. Thus, the hemolytic activity could employ an essential role for infections in *A.fumigatus*.

In the present study, with primers, F-Asphs and R-Asphs, an 180 bp fragment of *aspHS* gene were amplified for all tested *A. fumigatus* isolates. Two environmental isolates showed different pattern from other strains after cut with *Tag*I. Using primers AFhem1 and AFhem2, an 450 bp band fragment of *aspHS* gene was obtained for all 53 *A. fumigatus* isolates. Only one isolated demonstrated different pattern after cut with *NcoI.*

In conclusion, our results provide evidence

hemolysin activity and analysis of *aspHS* gene of *A. fumigatus*. These data may be useful in early diagnosis of *A. fumigatus* infections.

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