Study of Hemolysin Gene “aspHS” and Its Phenotype in Aspergillus Fumigatus

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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 which 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
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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 × 10⁶ from each A. fumigatus was inoculated to 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-isooamyl 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’-TGGTACAAAGGACGGTGACAA-3’) and R-Asphs (5’-GTCGCCAGTGGAATTCTGGCA-3’) for amplification of an 180 bp DNA [13] and Afhem1 (5’–GCATCGGTCCAAGCTTACGCA-3’) and Afhm2 (5’–TTAACAGTTGCAAATGGCACC-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 Tag 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. TagI (Fermentas; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for analysis of 180 bp
fragment was used. Ncol (Fermentas; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and TagI 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).

**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 | 6.6-6.5 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 | 2 | 24 | 12 | 31 | 39 | 100 |
| Total | 7 | 13 | 27 | 51 | 3 | 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).

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

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

**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**

**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](image)

Digestion of the aspHS gene products with Tagl 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 Tagl (Figure 5).

![Figure 5: Restriction fragment pattern of 450 bp A. fumigatus aspHS gene digested with Tagl. Lane M, 50 bp ladder; lane 1, IBRC-M30033; Lane 2, IBRC-M30040](image)

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

![Figure 6: Restriction fragment pattern of 450 bp A. fumigatus aspHS gene digested with Ncol. 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](image)

**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 Tagl. 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 Ncol.

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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