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ORIGINAL ARTICLE/ARTICLE ORIGINAL

Importance of elastase production in development of invasive aspergillosis

Importance de la production d'élastase dans le développement de l'aspergillose invasive

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KEYWORDS
Aspergillus fumigatus;
Aspergillosis;
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Pathogenicity

Summary
Objective. — To evaluate the relationship between elastase activity of different *Aspergillus fumigatus* (*A. fumigatus*) isolates and their pathogenicity in mice.
Material and methods. — The animals were intravenously (IV) infected with 1×10^6 conidia/mouse. The elastase activity in mice sera and survival time were determined. All animals were sacrified at the end of the fourth week, and autopsies were performed for histopathological examination. The tissue sections were stained with Periodic Acid Schiff (PAS) and Verhoeff's techniques.
Results. — The highest elastase activity was associated with the sera of infected mice with elastase-producing *A. fumigatus* AIR78 as standard strain (0.18 ± 0.02 mm), followed by infected animals with elastase-producing isolates (0.16 ± 0.02 mm) and control mice (0.14 ± 0.02 mm). The elastase activity index (EAI) was determined in value of 0.92 ± 0.27. The survival times in mice infected with elastase-producing isolate and in control group were 21.6 and 30 days, respectively. Histopathological studies showed that the heart and kidneys were more infected by *Aspergillus* hyphae than other tissues.
Conclusion. — It was concluded that there is a significant relationship between elastase activity and survival time in mice with aspergillosis.
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MOTS CLÉS
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Aspergillose ;
Élastase ;
Pathogénicité

Résumé
Objectif. — Évaluer la relation entre l'activité élastase de différents isolats d'*Aspergillus fumigatus* et leur pathogénicité.
Matériels et méthodes. — Les animaux ont été infectés par voie intraveineuse (IV) avec 1×10^6 conidies/souris. L'activité de l'élastase dans le sérum et le temps de survie des souris ont été déterminés. Tous les animaux ont été sacrifiés à la fin de la quatrième semaine, et les autopsies ont été effectuées pour examen histopathologique. Les coupes de tissus ont été colorées par l'acide périodique de Schiff (PAS) et les techniques de Verhoeff.
Résultats. — La plus forte activité élastase a été associée avec le sérum des souris infectées par la souche de référence d'*A. fumigatus* AIR78, productrice d'élastase ($0{,}18 \pm 0{,}02$ mm), suivie par les animaux infectés par les isolats producteurs d'élastase ($0{,}16 \pm 0{,}02$ mm) et souris témoins ($0{,}14 \pm 0{,}02$ mm). L'indice d'activité élastase (EAI) a été de $0{,}92 \pm 0{,}27$. Le temps de survie chez les souris infectées avec des isolats producteurs d'élastase et dans le groupe témoin étaient 21,6 et 30 jours, respectivement. Les études histopathologiques ont montré que le cœur et les reins étaient plus infectés par les hyphes (ou filaments) d'*A. fumigatus* que les autres tissus.
Conclusion. — Il a été conclu qu'il y a une relation significative entre l'activité de l'élastase et de temps de survie chez les souris avec aspergillose.
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Introduction

Aspergillus fumigatus (*A. fumigatus*) is a saprophytic, thermophilic and ubiquitous fungus in the environment. Because of the small size (<5 μm) and aerodynamic properties of *A. fumigatus* conidia, they can pass through the upper respiratory tract defenses and may reach distal regions of the lungs. In this region the host defenses rely on phagocytic cells to remove the conidia efficiently [19]. Inhalation of conidia of *A. fumigatus* can give rise to a number of different clinical forms of aspergillosis, depending on the immunologic status of the host [28]. Invasive aspergillosis is a common mycosis occurring in immunocompromised hosts undergoing cancer chemotherapy or immunosuppressive therapy following organ transplants [3]. Several reports suggest that the virulence of *A. fumigatus* is the result of the activity of numerous factors including adherence systems, toxins and extracellular enzymes [13,15,21]. Extensive colonization of lung tissue by *A. fumigatus* has suggested a key role for fungal proteases during infection. Among all proteases supposedly related to the pathogenicity of this fungus, elastase activity index (EAI) must be highlighted, not only because elastin has an essential role in the structure and physiology of the lungs [24], but also because this activity has been described in other important pathogens such as *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Bacteroides nodosus*, *Schistosoma* and some dermatophytes [26,27]. Elastase activity (EA) is well known for *A. fumigatus* and other species and occurs in at least 36 to 94% of all strains [8]. From a practical standpoint, elastase-producing strains promote a higher death rate in immunodeficient mice than non-elastase-producing strains. Alp and Arikan [1] investigated elastase activity in clinical isolates of *A. fumigatus*, *A. flavus* and *A. niger* and showed a possible correlation between the existence of this enzyme and development of invasive aspergillosis. Elastase activity was detected in 95.6%, 82.6%, and 0% of *A. fumigatus*, *A. flavus*, and *A. niger* isolates, respectively. Okumura et al. [17] have previously studied and reported the pathogenicity of elastase produced by *A. flavus* in mice immunocompromised with cyclophosphamide, cyclosporine and prednisolone. They demonstrated that severe lesions in bronchioli or alveoli are induced by elastase-producing strains in mice infected with *A. flavus*. According to the high ability of *A. fumigatus* isolates to produce elastase and to cause invasive aspergillosis, it was suggested that this enzyme is an important virulence factor. It was shown that the clinical isolates of *A. fumigatus* were more virulent than environmental strains [4]. However, not all the strains of *A. fumigatus* have the capacity to degrade elastin and to use this protein as source of nitrogen. Despite several previous reports showing occasional correlation between elastase activity in *Aspergillus* strains obtained from invasive mycoses, so far elastase activity has not been clearly linked to pathogenicity in *A. fumigatus* strains. The international collaboration effort are directed towards the improvement of our understanding of the virulence of this organism. The purpose of this study was to assess the role of elastase activity of *A. fumigatus* in the development of aspergillosis in murine model.

Materials and methods

Organisms

Twenty-six clinical isolates of *A. fumigatus* (12 isolates originated from human and 14 originated from animals with aspergillosis) and one elastase-producing *A. fumigatus* AIR78 (as standard strain) were obtained from Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran. The isolates were grown on Sabouraud dextrose agar containing chloramphenicol (0.005%; Merck Co., Darmstadt, Germany) at 37 °C for 5 days. Conidia were harvested with a 0.02% Tween 80 and washed twice in sterile saline.

Screening for elastase production

Conidia were cultured on agar containing 0.2% elastin (Sigma Chemical Co., St Louis, Mo, USA) and 0.01% yeast extract (Merck Co., Darmstadt, Germany) suspended in 0.5% Triton X-100 at 37 °C for 7—10 days. The hallo diameter (in

millimeter) surrounding fungal colony was considered as elastase production by fungus [6].

Production of elastase

Aspergillus fumigatus conidia were collected in sterile 0.1% Tween 80 and counted by hemacytometer to adjust 1×10^7 cells/mL. The amount of 1.5 mL of the conidial suspension was inoculated into 125 mL of liquid medium containing 1.17% yeast carbon base, 0.2% elastin and 0.3% $CaCo_3$. The cultures were incubated at 37 °C with shaking (100 rpm) for 5 days and then filtered through Whatman No. 114 filter paper. The cytoplasmic and cell wall extracts were prepared from *Aspergillus* hyphae [9].

Measurement of elastase activity

Cell free of broth filtrates and extracts were used for elastase activity assay using elastin-Congo red (Sigma Chemical Co., St Louis, Mo, USA) as the substrate. One milliliter of the assay buffer (10 mM sodium phosphate, pH = 7) was prepared and culture supernatant or porcine pancreatic elastase (Sigma Chemical Co., St Louis, Mo, USA) was added to assay buffer and rotated at 37 °C for 2 h. The absorbance of the supernatant was measured at 492 nm by enzyme-linked immunosorbent assay (ELISA) reader (Multiskan MS, Germany). Elastase activity of the fungal samples containing culture filtrate, cytoplasmic and cell wall extracts were determined by standard curve of known elastase activity. Sera were collected in four different times (days 0, 10, 20 and 30) for elastase activity before and after infection with *Aspergillus* conidia.

Experimental aspergillosis in mice

Conidia suspended in sterile saline were counted with hemacytometer and confirmed by culturing on Sabouraud dextrose agar (Merck Co., Darmstadt, Germany). Forty-two month-old, out bred, male mice were purchased from Pasteur institute of Iran and housed in four groups (10 mice each group) into the cages. Animals in group 1 were intravenously (IV) administered with sterile saline (control group), whereas animals in groups 2, 3 and 4 were IV infected with 1×10^6 conidia of elastase-producing *A. fumigatus* (originated from human with invasive aspergillosis; EAI: 1.73 and EA: 1.02), non-elastase producing *A. fumigatus* (originated from human with aspergilloma; EAI: 0.11 and EA: 0.01) and elastase-producing *A. fumigatus* AIR78, respectively. Body weight, food and water consumption were recorded every 3 days. Mortality and survival rates of infected mice were monitored for 4 weeks after inoculation.

Colony forming unit (CFU) and histopathological examination

One survivor of each mice group was sacrified after 4 weeks and different tissues, such as the brain, heart, kidneys, liver, lungs and spleen, were collected. One gram of each tissue was diluted with sterile saline (1:10) and homogenized (ELM homogenizer, Tehran, Iran). Subsequently, the suspensions were cultured on Sabouraud dextrose agar (Merck Co., Darmstadt, Germany) and incubated at 37 °C for 48 h. The number of fungal colonies were counted and expressed as colony forming unit (CFU). The histopathological examinations were applied for demonstrating the fungal invasiveness using Periodic Acid Schiff (PAS) and digestion of elastin using Verhoeff's staining techniques. Tissue sections were studied using light microscopy for degree of tissue damage, elastin in lung, artery necrosis and presence of fungal hyphae in tissues.

Statistical analysis

Chi-square, Student *t*-test and Wilcoxon—Mann—Whitney tests were used for statistical analysis of elastase activity and survival time in different groups. A *P* value less than 0.05 was statistically significant.

Results

Elastase activity

The results of fungal cultures on elastin medium showed that mean colony diameter and hallo diameter around isolates tested were 30.59 ± 11.92 mm and 34.5 ± 10.99 mm, respectively. The mean of hallo diameter/colony diameter (EAI) was calculated approximately 0.92 ± 0.027. Elastase activities on broth media were varied according to the source of *A. fumigatus* (Table 1). As shown in Table 2, the enzymatic activity in the sera of control mice (0.14 ± 0.02 mm) was lower than that of the infected animals with elastase-producing *A. fumigatus* AIR78 (0.18 ± 0.02 mm) and elastase-producing *A. fumigatus* isolates (0.16 ± 0.02 mm).

Survival time

The results indicated that all animals were alive in group 1, whereas the survival rates were calculated 50% in group 2, and 80% in groups 3 and 4. Significant difference was observed between group 2 and controls (group 1) during 30 days after infection ($P < 0.05$). The survival times in mice infected with elastase-producing isolate and in control group were 21.6 and 30 days, respectively.

Table 1 Elastase activity index (EAI) of *Aspergillus fumigatus* isolates in solid and broth media.
*Indice d'activité élastase des isolats d'*A. fumigatus *en milieu solide et en milieu liquide*.

Source	Number	Mean	SD
EAI			
Human	12	0.97	0.34
Animal	14	0.87	0.19
Standard	1	0.89	0.00
EA			
Human	12	0.21	0.02
Animal	14	0.18	0.04
Standard	1	0.15	0.00

EAI: Elastase Activity Index, Colony diameter/elastin zone diameter on solid medium; EA: Elastase activity on broth medium; Standard: Elastase-producing *A. fumigatus* AIR78; SD: Standard deviation.

Table 2 Elastase activity in mice sera after *Aspergillus fumigatus* conidia inoculation.
*Activité de l'élastase dans le sérum des souris après inoculation des conidies d'*Aspergillus fumigatus.

Group	No. of animals	Elastase activity (Mean ± SD)				Survival times (Days)	Mortality rate (%)
		Day 0	Day 10	Day 20	Day 30		
Control	10	0.117 ± 0.016	0.146 ± 0.027	0.164 ± 0.028	0.149 ± 0.041	30 ± 0.00	0
Elastase-producing isolate	10	0.157 ± 0.015	0.164 ± 0.028	0.199 ± 0.021	0.142 ± 0.008	21.6 ± 10.6	50
Non-elastase producing isolate	10	0.113 ± 0.016	0.188 ± 0.030	0.186 ± 0.011	0.123 ± 0.022	28.1 ± 4.01	20
Standard strain	10	0.158 ± 0.029	0.186 ± 0.032	0.199 ± 0.026	0.244 ± 0.015	25.7 ± 9.42	20

Standard strain: Elastase-producing *A. fumigatus* AIR78.

Table 3 Comparison of *Aspergillus fumigatus* cultures obtained from various tissues in different mice groups.
Comparaison des cultures d'Aspergillus fumigatus *obtenues à partir de divers tissus dans les différents groupes de souris.*

Group	Animal (No.)	Colony forming unit (CFU/g)					
		Brain	Heart	Kidney	Liver	Lung	Spleen
Control	1	0	0	0	0	0	0
Elastase-producing isolate	1	2111	3112	4122	1384	3407	2365
Non-elastase producing isolate	1	2113	3024	3875	1287	2989	2011
Standard strain	1	2750	3837	4396	1400	3412	2600

Colony Forming Unit and histopathological studies

A. fumigatus colonies were purely isolated from tissue culture of mice with aspergillosis. As shown in Table 3, no fungi were grown in culture media from control group. The results indicated that all tissues, except the brain, had more CFUs in mice infected with elastase-producing isolate than mice infected with non-elastase isolate. The highest fungal colonies were associated with the kidneys (4396 CFU/g), followed by the heart (3837 CFU/g), lungs (3412 CFU/g), brain (2750 CFU/g), spleen (2600 CFU/g) and liver (1400 CFU/g) in mice infected with standard strain. Histopathology of tissues showed a remarkable proliferation of fungal hyphae in brain and heart (Fig. 1). There was no microscopic or cultural evidence of infection in the tissues of control group. Elastin stained with Verhoeff's technique showed no difference between control group and other groups.

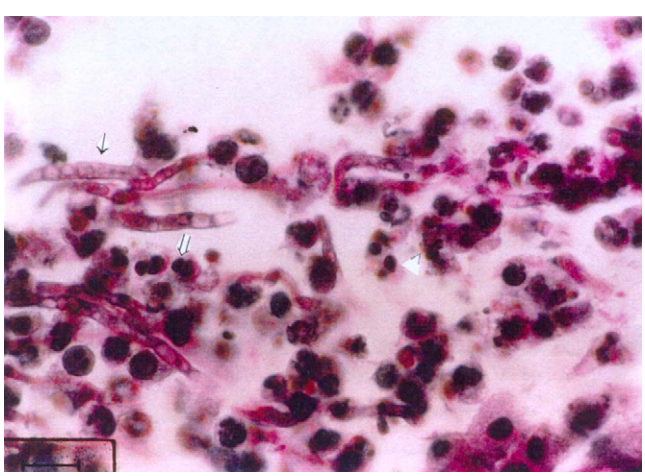

Figure 1 *Aspergillus fumigatus* hyphae in brain tissue of mice with invasive aspergillosis (PAS, 100×).
*Filaments d'*A. fumigatus *dans le tissu cérébral de souris atteintes d'aspergillose invasive (PAS, 100×).*

Discussion

We studied 26 clinical isolates of *A. fumigatus* for elastase activity on solid and broth medium containing elastin as a nitrogen substrate. In this study, EAI less than 0.92 for 16 clinical isolates and EAI more than 1 for 10 isolates were determined. In a previous study, the in vitro elastase activity of 198 strains of *A. fumigatus* obtained from clinical and environmental samples and the usefulness of this characteristic for predicting invasiveness with clinical isolates were evaluated [6]. A clear link between elastase activity and pathogenicity was demonstrated. Higher EAIs were related to strains isolated from invasive aspergillosis, whereas if EAI was less than 1, the probability of suffering from invasive disease was very low [6]. In another study, an association was demonstrated between EAI greater or equal to 1 and pathogenicity [2]. Some authors have suggested that elastase activity is an important, but not a determining factor in the pathogenicity of *A. fumigatus*. In a study of 38 isolates from patients with invasive aspergillosis in solid medium with elastin, Rhodes et al. [23] observed that all the isolates involved in the case of aspergillosis expressed elastase, but not all those that produced elastase were associated

to clinical aspergillosis. In addition, Garcia et al. [10] demonstrated that strains without elastase activity are capable of colonizing the lungs of experimentally infected mice. The ability to colonize the lungs enhanced the capacity to develop a higher elastase activity. This discrepancy may be due to the source of isolates. In addition, Debeaupuis et al. [7] were unable to discriminate between clinical and environmental isolates of 879 *A. fumigatus* strains with molecular typing. This fact has been interpreted as a proof of the hypothesis that any *A. fumigatus* strain is potentially pathogenic and could lead to a case of invasive aspergillosis if it encounters a susceptible host. Research to test these possibilities is in progress.

The elastase activity on broth medium is a better index to separate different isolates ($n = 26$) of *A. fumigatus* and also other species. This study showed that all of 26 isolates produced extracellular elastase with some variability among isolates (0.19 ± 0.04 unit/mL). Previous studies indicated elastase activity in 95.5% of *A. fumigatus* isolates using this method [1]. In a study conducted by Kolatukudy et al. [12], the extracellular elastionolytic proteases produced were 0.04 unit/mL by 13 isolates of *A. fumigatus* in elastin media. It was found that 9 of 13 strains of *A. fumigatus* demonstrated elastase activity greater than 1 unit/mL [16]; and *A. fumigatus* strain isolated from invasive pulmonary aspergillosis had more elastase activity than others [6]. The differences of elastase activity in broth medium between our results and other studies may be due to differences of wavelength. We used a wavelength of 492 nm whereas the results of other investigations were read at 550 or 402 nm [18,20].

Several studies have evaluated the relationship between proteolytic enzymes and pathogenicity [11,16,18]. Assessment of the effect of elastionolytic protease on rat showed that elastase-producing strain is more virulent than the non-protease producing strain [11,16]. In this study, high elastase-producing isolate was more virulent than the other ones. In addition, mortality rate in mice inoculated with elastase-producing strain of *A. fumigatus* (group 2) was higher than those of other groups. The results showed that the mortality rate was the same in groups 3 and 4 (20%) under study. It is not fully understood, but it is speculated that other virulence factors, such as the ability of the fungus in tissue conditions, thermotolerant action and different enzymes, are involved in the pathogenicity of different *A. fumigatus* strains. For this reason, this study should be continued to evaluate the exact virulence factors in tissue invasion and rate of mortality. Our result was in agreement with some studies showing that elastase play a key role in invasive aspergillosis [5,6,23]. It was shown that wild-type of *A. fumigatus* strains cause higher mortality rate than strains without protease activity in mice [12]. In a study by Okumura et al. [18], mortality rate decreased in deficiency mutant strains, whereas elastase-producing strains were more lethal. Aufauvre-Brown et al. [4] demonstrated that environmental strains were less virulent than their corresponding clinical strains. On the other hand, some studies showed no differences between elastase-producing strains and mutant isolates (elastase non-producing) in development of aspergillosis and suggested that all isolates of *A. fumigatus* are capable of causing infection [1,14,20,22]. These differences may be due to the approach of infection; in some studies mice were infected through inhalation [4,11,18], whereas in this study mice were infected intravenously. Other reasons are associated with genetic variability in strains and the numbers of conidia used for infection [7,16,20].

The results of tissue culture from animals revealed that this method was suitable and that fungus are able to grow on medium. The histopathological studies showed fungal hyphae and inflammatory cells in different tissues such as brain and heart. These results can be related to degradation of heterologous protein by elastase function against elastin in some tissues [25]. Neutrophil infiltrations and focal hemorrhagic lesions were observed in the alveolar spaces in many areas of the lungs and also septal edema. The comparison of histopathology to CFU revealed that histopathological analysis could confirm the presence of invasive aspergillosis, but histology has limitations since examination of whole organ is not practicable, and histological examination of the small sections were negative; although it suggested that CFU in lung tissue culture is more sensitive than survival time [4]. In summary, this study showed that elastase activity of *A. fumigatus* could be one of the important virulence factors in aspergillosis in murine model and indicated differences in the mortality rate in mice between elastase-producing strains and non-elastase-producing strains.

Disclosure of interest

The authors have not supplied their declaration of conflict of interest.

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References

[1] Alp S, Arikan S. Investigation of extracellular elastase, acid proteinase and phospholipase activities as putative virulence factors in clinical isolates of *Aspergillus* species. J Basic Microbiol 2008;48:331—7.

[2] Alvarez-Perez S, Blanco JL, Alba P, Garcia ME. Mating type and invasiveness are significantly associated in *Aspergillus fumigatus*. Med Mycol 2009;26:1—6.

[3] Annaisie EJ, McGinnis MR, Pfaller MA. Clinical mycology. Philadelphia USA.: Churchill-Livingstone; 2003. pp. 273—296.

[4] Aufauvre-Brown A, Brown JS, Holden DW. Comparison of virulence between clinical and environmental isolates of *Aspergillus fumigatus*. Eur J Clin Microbiol Infect Dis 1998;17:778—80.

[5] Blackman MJ. Proteases involved in erythrocyte invasion by the malaria parasite: function and potential as chemotherapeutic targets. Curr Drug Targets 2000;1:59—83.

[6] Blanco JL, Hontecillas R, Bouza E, Blanco I, Pelaez T, Munoz P. Correlation between the elastase activity index and invasiveness of clinical isolates of *Aspergillus fumigatus*. J Clin Microbiol 2002;40:1811—3.

[7] Debeaupuis JP, Sarafati J, Chazalet V, Latge JP. Genetic diversity among clinical and environmental isolates of *Aspergillus fumigatus*. Infect Immun 1997;65:3080—5.
[8] Denning DW, Elliot J, Keaney M. Temperature-dependent expression of elastase in *Aspergillus* species. J Med Vet Mycol 1993;31:455—8.
[9] Frosco M, Chase TJR, Macmillan J. Purification and properties of the elastase from *Aspergillus fumigatus*. Infect Immun 1992; 60:728—34.
[10] Garcia ME, Caballero J, Blanco I, Cruzado M, Costas E, Blanco JL. Changes in the elastase activity and colonization ability of *Aspergillus fumigatus* after successive inoculations in mice. Rev Iberoam micol 2006;23:221—3.
[11] Hasegawa Y, Nikai T, Shibata Y, Komori Y, Homma M, Ogawa K, et al. The role of virulence factor of elastolytic proteinase from *Aspergillus fumigatus*. Nippon Ishinkin Gakkai Zasshi 1999; 40:85—91.
[12] Kolatukudy PE, Lee JD, Rogers LM. Evidence for possible involvement if an elastinolytic serine protease in aspergillosis. Infect Immun 1993;61:2357—68.
[13] Latgé JP. The pathobiology of *Aspergillus fumigatus*. Trends Microbiol 2001;9:382—9.
[14] Nierman WC, Pain A, Anderson MJ, Wortman JR, Kim HS, Arroyo J. Genomic sequence of the pathogenic and allergenic filamentous *Aspergillus fumigatus*. Nature 2005;438:1151—6.
[15] Odds FC, Gow NAR, Brown AJP. Fungal virulence studies come of age. Gen Biol 2001;2:1009 [1-9. 4].
[16] Okumura Y, Ogawa K, Nikai T. Elastase and elastase inhibitor from *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus niger*. J Med Microbiol 2004;53:351—4.
[17] Okumura Y, Ogawa K, Nikai T. Pathogenicity of elastase from *Aspergillus flavus*. J Res Inst Meijo Univ 2007;6:145—52.
[18] Okumura Y, Ogawa K, Uchida K, Komori Y, Nonogaki T, Nikai T. Biological properties of elastase inhibitor AFLEI from *Aspergillus flavus*. Jpn J Med Mycol 2008;49:87—93.
[19] Okumura Y, Ogawa K, Uchiya K, Nikai T. Characterization and primary structure of elastase inhibitor AFLEI from *Aspergillus flavus*. Jpn J Med Mycol 2007;48:13—8.
[20] Reeves EP, Messina CGM, Doyle S, Kavanagh K. Correlation between gliotoxin production and virulence of *Aspergillus fumigatus* in *Galleria mellonella*. Mycopathologia 2004;158: 73—9.
[21] Rementerıa A, Lopez-Molina N, Ludwig A. Genes and molecules involved in *Aspergillus fumigatus* virulence. Rev Iberoam Micol 2005;22:1—23.
[22] Reichard U, Buttner S, Eiffert H, Staib F, Ruchel R. Purification and characterization of an extracellular serine proteinase from *Aspergillus fumigatus* and its detection in tissue. J Med Microbiol 1990;33:243—51.
[23] Rhodes JC, Bode RB, McCuan-Kirsch CM. Elastase production in clinical isolates of *Aspergillus*. Diagn Microbiol Infect Dis 1998;10:165—70.
[24] Starcher BC. Lung elastin matrix. Chest 2000;117:229S—34S.
[25] Ward OP, Qin QM, Dhanjoon J, Ye J, Singh A. Physiology and biotechnology of *Aspergillus*. Adv Appl Microbiol 2006;58:1—75.
[26] Werb Z, Banda MJ, McKerrow JH, Sandhaus RA. Elastases and elastin degradation. J Invest Dermatol 1982;79:154s—9s.
[27] Woods DE, Cryz SJ, Friedman RL, Iglewski BH. Contribution of toxin A and elastase to virulence of Pseudomonas aeriuginosa in chronic lung infections of rats. Infect Immun 1982;36: 1223—8.
[28] Xess I, Mohanty S, Jain N, Banerjee U. Prevalence of *Aspergillus* species in clinical samples isolated in an Indian tertiary care hospital. Indian J Med Sci 2004;58:513—9.