Mating type and invasiveness are significantly associated in *Aspergillus fumigatus*

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In recent years, several lines of evidence have questioned the asexual nature of *Aspergillus fumigatus*, showing that this fungus possesses a fully functional sexual reproductive cycle that leads to the production of cleistothecia and ascospores. The presence of a sexual cycle in *A. fumigatus* could have significant medical implications, as sexual reproduction might contribute to increased virulence or resistance to antifungal agents. In the present work, we studied the relationship between mating type and invasiveness in *A. fumigatus*. Statistical analysis of the results showed a significant association between the mating type MAT1-1 and an invasive origin of the isolates. Similarly, when the clinical or environmental origin of isolates was considered instead of their invasive or non-invasive origin, a significant association between the mating type MAT1-1 and clinical origin was observed. Finally, the association between mating type MAT1-1 and pathogenicity, measured by an Elastase Activity Index (EAI), was significant. Our results suggest a possible association between the MAT1-1 mating type and *A. fumigatus* invasiveness.

**Keywords** *Aspergillus fumigatus*, mating type, invasiveness, Elastase Activity Index

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**Introduction**

*Aspergillus fumigatus* is an opportunistic fungal pathogen that has long been considered asexual, with dispersal achieved by the abundant production of haploid conidia [1–3]. However, in recent years, several lines of evidence have questioned this assumption (reviewed in [1]). Genomic screening of *A. fumigatus* resulted in the identification of several genes associated with different stages of the sexual cycle in ascomycete fungi, including the mating process, pheromone response, meiosis and fruiting body development [4–7]. Moreover, some of the sex-related genes, including those involved in mating compatibility (*MAT1-1* and *MAT1-2*), are expressed during mycelial growth of *A. fumigatus* [8].

The ultimate proof that *A. fumigatus* maintains its sexual potential has been provided by O’Gorman et al. [9] who found that *A. fumigatus* possesses a fully functional sexual reproductive cycle that leads to the production of cleistothecia and ascospores. The teleomorph of *A. fumigatus* was assigned to the genus *Neosartorya* on the basis of phylogenetic relatedness and morphology of sexual structures, and named *Neosartorya fumigata* O’Gorman, Fuller & Dyer *sp. nov.* [9]. The presence of a sexual cycle in *A. fumigatus* has significant medical implications, as sexual reproduction might result in increased virulence or resistance to antifungal agents [1,9].

*A. fumigatus* virulence is considered to be multifactorial [2,3,10–12]. Among the multiple factors supposedly related to pathogenicity in this mould, the elastase activity is one of the most important. Elastin has an essential role in the structure and physiology of the lung [13], and elastase activity has been described in other important pulmonary pathogens [14]. Therefore, investigating the potential link between the mating type of *A. fumigatus* isolates with invasiveness, through the possible intervention of elastase activity, is highly relevant.

The aim of this work was to study the relationship between mating type and invasiveness in *A. fumigatus*. Mating type and the elastase activity index (EAI) were
determined in a sample of 102 clinical and environmental isolates. In addition, the possible relationship between mating type and other 19 extracellular enzymatic activities was also studied.

Materials and methods

Fungal isolates

A total of 102 fungal isolates from our culture collection, 57 of clinical origin and 45 isolated from the environment, were used in this work. Twenty-eight isolates were recovered from patients with clinical signs of invasive aspergillosis (IA) which was later confirmed as IA. Alternatively, 23 strains (colonizers) were isolated from patients without clinical evidence of aspergillosis and who did not develop IA. The final six clinical isolates were from patients with aspergillum (fungus ball). All of these strains were kindly provided by the Hospital General Universitario Gregorio Marañón (Madrid, Spain). The environmental isolates were collected in different wards of this hospital.

All isolates had been previously identified as *Aspergillus fumigatus* on the basis of their macroscopic and microscopic characteristics.

EAI determination

The EAI was determined as described previously [15]. Briefly, the 102 isolates were cultured on Sabouraud dextrose agar (bioMérieux, Marcy l’Etoile, France) and incubated at 37°C until abundant sporulation was observed. After which, a loopful of an Aspergillus conidial suspension (10⁶ conidia/ml) was inoculated at a central point of a Petri dish containing the medium described by Kothary et al. [16]. Elastin plates were incubated at 37°C for 10 days before determining the EAI as the ratio between the diameters of the halo of elastin lysis which were easily observed and the fungal colonies.

Determination of other enzymatic activities

Nineteen enzymatic activities were assayed for a representative sample of 55 of the 102 *A. fumigatus* isolates using API-ZYM strips (bioMérieux). This is a semi-quantitative method that allows for the assay of 19 different extra-cellular enzymes. Each of the 55 isolates was grown in Czapek-Dox broth (Pronadisa, Madrid, Spain) at 37°C for 24 h in aerated culture. During this time, the conidia germinated to form minute granular colonies. Portions of the liquid (65 μl) were taken directly from these cultures and used to inoculate each cupule of the API-ZYM strips which were then incubated at 37°C for 4 h. After this time, color reagents Zym A and Zym B were added as recommended by the supplier and the strips read in accord with a color chart provided by the manufacturer. Enzymatic activities were scored on a semi-quantitative scale ranging from 0 (negative reaction) to 5 (maximum intensity) with 1 to 5 considered as positive results. Czapeck-Dox broth was used as a negative control.

Genomic DNA isolation

A modification of the methodology described by Liu et al. [17] was used. Briefly, 500 μl of lysis buffer (400 mM Tris-HCl [pH 8.0], 60 mM EDTA [pH 8.0], 150 mM NaCl, 1% SDS p/v) were added to a microcentrifuge tube containing the inoculum (a loopful of *Aspergillus* sporulated mycelium from plate cultures). The tubes were vortexed briefly and incubated for 2 h at 65°C. After adding 150 μl of potassium acetate pH 4.8, made as 6 ml of 5 M potassium acetate, 1.15 ml of glacial acetic acid and 2.85 ml of distilled water, the tubes were vortexed again and centrifuged at 8000 g for 5 min. The supernatants were transferred to new tubes and centrifuged as described above. Each supernatant was transferred to another microcentrifuge tube and an equal volume of 2-isopropanol (Sigma-Aldrich, Madrid, Spain) was added. The tubes were mixed by inversion and centrifuged at 16000 g for 15 min to precipitate the DNA. DNA pellets were washed with 300 μl of 70% cold ethanol and centrifuged at 16,000 g for 10 min to eliminate the supernatant. DNA precipitates were dried at 37°C to remove all traces of ethanol and resuspended in 150 μl of sterilized distilled water.

Mating type determination

To determine the mating type of the fungal isolates, the multiplex PCR described by Paoletti et al. [8] was adapted to our laboratory conditions. This PCR assay includes a MAT1-1 specific primer, AFM1 (5′-CCTTTGACCGGATGGGGTG-3′), and a MAT1-2 specific primer, AFM2 (5′-CGCTCCTCAGAACAATCG-3′), together with a common primer, AFM3 (5′-CGGAAATCTGATGTCGCC-3′) flanking the MAT locus [8]. PCR reactions were performed using the PureTaq Ready-To-Go PCR Beads kit (Amersham Biosciences, Buckinghamshire, UK). Reaction mixtures contained 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, 200 μM of each dNTP and 2.5 U puReTaq DNA polymerase. Ten microliters of DNA extract containing the inoculum (a loopful of mycelium from plate cultures). The tubes were vortexed briefly and incubated for 24 h at 65°C. The tubes were then incubated at 37°C for 4 h. After this time, color reagents Zym A and Zym B were added as recommended by the supplier and the strips read in accord with a color chart provided by the manufacturer. Enzymatic activities were scored on a semi-quantitative scale ranging from 0 (negative reaction) to 5 (maximum intensity) with 1 to 5 considered as positive results. Czapeck-Dox broth was used as a negative control.

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at 60°C and 1 min at 72°C, and a final extension of 5 min at 72°C.

Ten microliters of each PCR product were electrophoresed on a 1.6% agarose gel. Gels were stained with ethidium bromide and visualized under UV light. Digitalized images of the gels were captured with a Gelprinter image analyzer (TDI, Madrid, Spain). The mating type of each isolate was determined according to the size of the amplification product: 834 base pairs (bp) for MAT1-1 and 438 bp for MAT1-2 [8], using a marker (GeneRuler 100bp DNA Ladder, Fermentas).

**Statistical analysis of results**

To analyze the possible existence of statistically significant associations between variables, a chi-square test was performed using statistical software (Statgraphics Plus 5.0 for Windows, Statistical Graphics Corp.). Yates’ correction was applied when needed. *P*-values <0.05 were considered significant.

**Results**

The distribution of the 102 *A. fumigatus* isolates used in this study is presented in Table 1 according to their mating type, origin and EAI results. As seen in the table, 58 isolates (56.9%) were of mating type MAT1-1, whereas the remaining 44 (43.1%) were MAT1-2. In no instances were both mating-type bands amplified from a single isolate. As an example, the results of mating type determination by PCR for 20 of the 102 isolates are shown in Fig. 1.

The distribution of mating types by origin of the isolates was remarkable because 22 of the 28 strains isolated from cases of invasive aspergillosis (IA) were MAT1-1. In contrast, the ratio of mating types among strains of other origin was close to 1:1 (36 MAT1-1: 38 MAT1-2). The statistical analysis of these results showed a significant association between the mating type MAT1-1 and the invasive origin of isolates (corrected $\chi^2 = 6.25$, *P* = 0.0125). Similarly, when the clinical or environmental origin of isolates was considered instead of their invasive or non-invasive origin, the association between the mating type MAT1-1 and clinical origin was also significant (corrected $\chi^2 = 4.20$, *P* = 0.0405).

With respect to the distribution of mating types according to their EAI, 69.8% of the isolates that showed an EAI $\geq 1$ were MAT1-1, while among the isolates with an EAI <1, the ratio of mating types was approximately 1:1 (28 MAT1-1: 31 MAT1-2). When the results were statistically analyzed, the association between mating type MAT1-1 and the possession of an EAI $\geq 1$ was significant (corrected $\chi^2 = 4.18$, *P* = 0.0409).

The distribution of mating types according to the presence or absence of 19 extracellular enzymatic activities is shown in Table 2. In contrast to the result observed for elastase activity, no association could be established between mating type and any enzymatic activity determined by the API-ZYM system. In fact, some enzymatic activities (lipase C14, cystine arylamidase, trypsin, chymotrypsin, β-glucuronidase and α-fucosidase) were not detected in any of the 55 isolates studied. However, all the isolates of both mating types presented the following three enzymatic activities: esterase C1, β-glucosidase and N-acetyl-β-glucosaminidase.

**Discussion**

Formerly, most fungal species of clinical importance were considered asexual but the ‘genomic revolution’ has enabled the identification of genomic sex-determining structures in some of these species [18]. Several attempts have been made to find a relationship between mating types and virulence of the main fungal pathogens. For example, Kwon-Chung et al. [19] demonstrated, using congenic strains of *Cryptococcus neoformans* var. *neoformans* (serotype D), that the $\alpha$ mating type is more virulent than the $\alpha$ type. No difference in virulence between mating types has been found with *C. neoformans* var. *grubii* (serotype A), but cells of the $\alpha$ type have been shown to have an enhanced predilection to penetrate the central nervous system during co-infection experiments in mice [20]. Furthermore, an uneven distribution of mating types has been described in clinical but not environmental isolates of other important pathogenic fungi, such as *Histoplasma capsulatum* [21].

The relationship between mating type and virulence in *A. fumigatus* has not yet been addressed. This might be
due, at least in part, to the more even distribution of mating types observed in *A. fumigatus* than in *C. neoformans* in which the α mating type predominates [18,22,23]. For example, in a sample of 290 *A. fumigatus* isolates, Paoletti et al. [8] observed that the MAT1-1 and MAT1-2 genotypes were represented in a similar proportion, both in environmental and clinical isolates. The latter strains were recovered from cases of IA, aspergilloma, colonizations and even animal aspergillosis. Another recent study reported a higher proportion of MAT1-2 isolates, resulting in a ratio close to 1:2 [24]. However, none of these works differentiated the clinical isolates according to their origin in the statistical analysis of the results. In the present study, after grouping the 57 clinical isolates according to their origin (IA, aspergilloma or colonization), we observed an almost four-fold higher frequency of MAT1-1 than MAT1-2 genotypes among isolates from cases of IA. Finally, among non-invasive strains of other origin, both mating types were represented in essentially the same proportion. These results suggest the possible existence of an association between the MAT1-1 mating type and the invasiveness of *A. fumigatus*.

The virulence of *A. fumigatus* is considered to be the result of numerous factors, including adherence systems, toxins and extracellular enzymes [2,3,10–12]. However, to date, no studies have investigated a link between mating type and *A. fumigatus* virulence. In this work, we studied this relationship indirectly, by determining the possible association between mating type and activity of several extracellular enzymes. Our main finding was that, in the sample studied, a statistically significant association exists between mating type and elastase activity, as a wide majority of *A. fumigatus* isolates that presented an EAI/H11350 were MAT1-1.

An important factor to consider is that the isolation of a particular strain from an infectious process does not mean that it is the etiologic agent. As has been recently demonstrated in our laboratory, different strains of *A. fumigatus* showing different combinations of mating type and EAI can coexist in patients suffering from IA [25]. Therefore, it might be difficult to identify the strain responsible for the process, or if a mixed infection has been established.

The role of elastase activity in the pathogenesis of IA has been widely discussed [26]. Although some authors

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**Table 2** Distribution of mating types in a sample of 55 *Aspergillus fumigatus* isolates according to the presence or absence of 19 enzymatic activities

<table>
<thead>
<tr>
<th>Enzymatic activity</th>
<th>MAT1-1</th>
<th>MAT1-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+*</td>
<td>−**</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>Esterase (C1)</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>Esterase lipase (C8)</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td>Lipase (C14)</td>
<td>0</td>
<td>34</td>
</tr>
<tr>
<td>Leucine arylamidase</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td>Valine arylamidase</td>
<td>1</td>
<td>33</td>
</tr>
<tr>
<td>Cystine arylamidase</td>
<td>0</td>
<td>34</td>
</tr>
<tr>
<td>Trypsin</td>
<td>0</td>
<td>34</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>0</td>
<td>34</td>
</tr>
<tr>
<td>Acidic phosphatase</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>Naphthol-AS-BI-phosphohydrolase</td>
<td>33</td>
<td>1</td>
</tr>
<tr>
<td>α-Galactosidase</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>7</td>
<td>27</td>
</tr>
<tr>
<td>α-Glucoronidase</td>
<td>0</td>
<td>34</td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>10</td>
<td>24</td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>N-acetyl-β-glucosaminidase</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>α-Mannosidase</td>
<td>1</td>
<td>33</td>
</tr>
<tr>
<td>α-Fucosidase</td>
<td>0</td>
<td>34</td>
</tr>
</tbody>
</table>

*Number of isolates of this mating type that show the enzymatic activity.

**Number of isolates of this mating type that do not show the enzymatic activity.
claim that there is no proof that any protease of *A. fumigatus* plays an essential role in the pathogenesis of IA [2], our previous work demonstrated a strong correlation between elastase activity, expressed as the EAI, and the invasiveness of this mould [15]. Furthermore, we have observed that after consecutive inoculations in mice, *A. fumigatus* strains may increase their elastase activity [27]. In the present work we have found not only the higher prevalence of MAT1-1 mating type among *A. fumigatus* isolates of invasive origin, but also the existence of a statistically significant association between this mating type and an EAI ≥1.

In conclusion, this is the first report demonstrating a significant association between mating type and the pathogenicity of *A. fumigatus*. This association should be confirmed in animal models. Also, further investigation on the possible link between mating type and other relevant traits of this mould is necessary.

**Acknowledgements**

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