การศึกษายีน mating type และความสัมพันธ์กับการสร้าง biofilm ของเชื้อ *Aspergillus fumigatus* จากสิ่งส่งตรวจจากโรงพยาบาลศิริราชและโรงพยาบาลสงขลานครินทร์

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Study on mating type and association with pathogenesis of *Aspergillus fumigatus* from clinical isolates collected at Siriraj hospital and Songklanagarind hospital

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บทคัดย่อ

การสืบพันธุ์แบบอาศัยเพศของเชื้อราหลายชนิดในสกุล *Aspergillus* ได้ถูกค้นพบเมื่อไม่นานมานี้ การค้นพบคุณสมบัติของการสืบพันธุ์แบบอาศัยเพศดังกล่าวก่อให้เกิดคำถามถึงบทบาทสำคัญของการสืบพันธุ์แบบอาศัยเพศต่อการอยู่รอดของเชื้อจุลชีพนี้ ตลอดจนผลกระทบอย่างมีนัยสำคัญทางการแพทย์ อาทิเช่น การนำไปสู่ความรุนแรงในการก่อโรคที่มากขึ้นหรือมีความดื้อต่อยาต้านเชื้อรามากขึ้น ในบรรดาเชื้อราในตระกูล *Aspergillus* พบว่า *Aspergillus fumigatus* เป็นเชื้อราฉวยโอกาสที่พบว่ามีอุบัติการณ์สูงสุดในผู้ป่วยภูมิคุ้มกันบกพร่องโดยเฉพาะอย่างยิ่งในผู้ป่วยที่มีภาวะบกพร่องเม็ดเลือดขาวชนิด neutrophil (neutropenia) ซึ่งการสืบพันธุ์แบบอาศัยเพศของ *A. fumigatus* นี้ มียีนที่เกี่ยวข้องอยู่สองยีน ได้แก่ยีน mating type 1-1 (*MAT1-1*) และยีน mating type 1-2 (*MAT1-2*) และต้องอาศัยกระบวนการ crossing ระหว่างสายพันธุ์ที่มีชนิดของยีน mating type ที่ต่างกัน ที่น่าสนใจคือ จากการศึกษาที่ผ่านมาพบว่า ชนิดของยีน *MAT* มีความสัมพันธ์อย่างมีนัยสำคัญกับพยาธิกำเนิด เนื่องจากมีปัจจัยก่อโรค (virulence factor) หลายปัจจัยที่ทำให้เชื้อ *A. fumigatus* มีความสามารถในการก่อโรคในมนุษย์ได้ อาทิเช่น ความสามารถในการเกาะติดกับเซลล์เยื่อบุถุงลมปอด ความสามารถในการลบหลีกภูมิคุ้มกันและความสามารถในการสร้างไบโอฟิล์มในปอดเป็นต้น ดังนั้นการศึกษาในครั้งนี้ จึงมีวัตถุประสงค์เพื่อตรวจสอบชนิดของยีน mating type ในเชื้อ *A. fumigatus* จากสิ่งส่งตรวจที่ได้รับจากโรงพยาบาลศิริราชและโรงพยาบาลสงขลานครินทร์ ตลอดจนเปรียบเทียบความสัมพันธ์ระหว่างชนิดของยีน *MAT* กับพยาธิกำเนิดของเชื้อ *A. fumigatus* ซึ่งการศึกษาในครั้งนี้ ได้เน้นไปที่ความสามารถในการสร้างไบโอฟิล์มของเชื้อราสายพันธุ์นี้ ในการศึกษาครั้งนี้ได้ทำการระบุสปีชีย์ของเชื้อ *Aspergillus* ที่ได้จากสิ่งส่งตรวจด้วยวิธีตรวจสอบสัณฐานวิทยาของเชื้อและทำการยืนยันสปีชีย์ด้วยการหาลำดับเบสของดีเอ็นเอ (DNA sequencing) จากนั้นทำการตรวจสอบชนิดของยีน *MAT* ด้วยปฏิกิริยาลูกโซ่โพลิเมอเรส (polymerase chain reaction) และทำ biofilm formation assay เพื่อศึกษาความสัมพันธ์ระหว่างชนิดของยีน *MAT* กับการสร้างไบโอฟิล์ม ผลจากการศึกษาสัณฐานวิทยาและ DNA sequencing แสดงว่าเชื้อราที่ได้รับจากสิ่งส่งตรวจจำนวน 23 ตัว เป็นเชื้อ *A. fumigatus* ซึ่ง 5 ตัว มียีนชนิด *MAT1-1* ในขณะที่ 18 ตัว มียีนชนิด *MAT1-2* ผลของ biofilm formation assay พบว่าไม่มีความแตกต่างอย่างมีนัยสำคัญระหว่างการสร้างไบโอฟิล์มของเชื้อ *A. fumigatus* ที่มียีน *MAT1-1* และยีน *MAT1-2* ผลของการทดลองในครั้งนี้แสดงให้เห็นว่าไม่มีความสัมพันธ์กันระหว่างชนิดของยีน *MAT* กับการสร้างไบโอฟิล์มของเชื้อ *A. fumigatus* อย่างไรก็ตามการศึกษาต่อไปเกี่ยวกับความสัมพันธ์ระหว่างยีน *MAT* กับปัจจัยก่อโรคอื่น ๆของเชื้อ *A. fumigatus* มีความสำคัญที่จะทำให้เข้าใจพยาธิกำเนิดของจุลชีพนี้มากขึ้น

คำสำคัญ: *Aspergillus fumigatus* ไบโอฟิล์ม ปัจจัยก่อโรค พยาธิกำเนิด

Abstract

In recent years, a sexual cycle of several fungal species in the genus *Aspergillus* were described. The description of heterothallic sexuality in *Aspergillus* species raised questions such as the role of sexual reproduction in the survival of this microorganism and whether it could have significant medical implications, such as contribution to increased virulence or increased antifungal drug resistance. Among species in the genus *Aspergillus*, *Aspergillus fumigatus* is the most common cause of opportunistic fungal infection in immunocompromised host, especially in those with neutropenia. Sexual reproduction in this species is governed by two different mating-type genes, mating-type 1-1 gene (*MAT1-1*) and mating-type 1-2 gene (*MAT1-2*), and requires a crossing between isolates of different mating-type gene.Interestingly, a significant association between the type of *MAT* gene and pathogenesis has been recently reported. As there are several virulence factors that contribute to ability of *A. fumigatus* to cause diseases in human, such as ability to adhere to the mucus membrane of alveoli, ability to evade host immunity and ability to form biofilm in the lung, . Thus, this study aimed to determine the mating type gene of *A. fumigatus* from clinical isolates collected at Siriraj Hospital and Songklanagarind Hospital and to study the relationship between gene *MAT* and pathogenicity of *A. fumigatus*, focused on biofilm formation. In this study, phenotypic examinations were performed to identify the species of *Aspergillus* from clinical isolates and confirmed species identification by DNA sequencing. Polymerase chain reaction (PCR) was performed to determine mating type gene and biofilm formation assay was carried out to study the relationship between the gene *MAT* and biofilm formation. The result from phenotypic method and DNA sequencing revealed that 23 isolates were identified as *A. fumigatus* by. Among them, 5 isolates revealed *MAT1-1* type, while 18 isolates were of *MAT1-2* type. The result of the biofilm formation assay showed no statisticallysignificance difference between *A. fumigatus MAT1-1* and *MAT1-2*. This result suggests that there is no relationship between mating type gene and biofilm formation. Further study about relationship between *MAT* gene and other virulence factors of *A. fumigatus* are important for better understanding pathogenesis of this microorganism.

Keywords: *Aspergillus fumigatus*, biofilm, virulence factor, pathogenesis

Introduction

Aspergillus species are commonly found saprophytic fungi that are important causes of infection among patients with immunodeficiency or patients receiving immunosuppressive agents. Infection begins with inhalation of airborne conidia, which are widely distributed in the environment. Once the conidia reach the alveoli of susceptible hosts, they initiate the germination process to produce angioinvasive hyphae that can invade tissue and endothelial cells, causing severe tissue necrosis.

The ascomycetes *Aspergillus* species have been previously regarded as asexual fungi. Recent genome analysis (Nierman et al., 2005; Mogensen, Nielsen, Hofmann, & Nielsen, 2006), however, revealed that the genome of certain *Aspergillus* species includes mating-type (*MAT*) locus and a set of genes required for sexual reproduction. In ascomycetes, a sexual reproduction is governed by two different mating-type genes, mating-type 1-1 gene (*MAT1-1*) and mating-type 1-2 gene (*MAT1-2*). The mating type gene *MAT1-1* encodes a protein with the so-called α box domain, whereas the gene product of *MAT1-2* contains a high mobility group (HMG) domain. In homothallic fungi, both *MAT1-1* and *MAT1-2* are present, while in heterothallic fungi, only one mating-type gene is required for a crossing between isolates of opposite mating-type.

In *Aspergillus fumigatus*, the most common pathogenic member in the genus *Aspergillus*, genome-wide screening revealed 215 genes implicated in sexual development, including the high-mobility group (HMG)-domain gene which is typically required for sexual reproduction (Mogensen et al., 2006), suggesting that *Aspergillus fumigatus* may be capable of sexual reproduction. Later on, sexual cycle of *A. fumigatus* was discovered and sexual reproduction was successfully induced (O'Gorman, Fuller, & Dyer, 2009).

Furthermore, *MAT* genes have also been identified in many other *Aspergillus* species including *A. oryzae*, *A. nomius*, *A. caelatus*, *A. tamarii*, *A. sojae*, *A. bombycis*, *A. niger*and *A. clavatus* (Kwon-Chung & Sugui, 2009; Pal, 2007). This finding indicates possible significance of the sexual reproduction. Classification of the teleomorph (sexual stage) of the genus *Aspergillus* is based on the presence of cleistothecia, ascospores and the phylogenetic relatedness, such as *Neosartoya fumigata* and *Petromyces flavus* being the teleomorph of *Aspergillus fumigatus* (O'Gorman, 2009) and *Aspergillus flavus* (Ramirez-Prado, Moore, Horn, & Carbone, 2008), respectively.

Recent discovery of sexual reproduction in *Aspergillus* spp. has initiated exciting discussion regarding significance of fungal sexual reproduction, such as whether the progeny from sexual reproduction will have increased virulence or increased resistance to antifungal agent (O'Gorman et al., 2009). The existence of two different mating types in the genome of *Aspergillus* spp. (either *MAT1-1* or *MAT1-2*) also raises the question about association between mating type and pathogenesis of *Aspergillus* spp.

Interestingly, the mating type of the opportunistic yeast *Cryptococcus neoformans* was shown to be associated with virulence (Kwon-Chung, Edman, & Wickes, 1992). This discovery raises the question of whether such relationship between mating type and virulence also exist in *Aspergillus* spp. In 2010, Alvarez-Pérez et al. indeed observed a relationship between mating type and invasiveness in *Aspergillus fumigatus*, whereby *MAT1-1* was shown to be significantly associated with elastase activity (Galagan et al., 2005). As elastase promotes tissue invasion and serves as important virulence factor in several pathogenic microorganism, it is possible that *Aspergillus* spp. with *MAT1-1* gene may reveal higher virulence than those with *MAT1-2*. In addition, Cheema & Christians reported that virulence of *A. fumigatus* in the larvae of the wax moth *Galleria mellonella* as an *in vivo* model was different among the mating types (Cheema & Christians, 2011). The survival rate of *Galleria mellonella* larvae injected with *MAT1-1 Aspergillus fumigatus* isolates was lower than those injected with *MAT1-2* isolates, suggesting that variation concerning virulence may exist between different mating types.

Several virulence factors have been previously described in *Aspergillus* spp., including capability to form biofilm (Abad, 2010). For better understanding the pathogenesis of aspergillosis, this study aimedto determine the mating type gene of *Aspergillus fumigatus* clinical isolates collected at Department of Microbiology, Siriraj Hospital and Songklanagarind Hospital and to investigate association with biofilm formation.

Materials and methods

Fungal isolates and culture conditions

Fungal clinical isolates obtained from Siriraj Hospital and Songklanagarind Hospital that were identified by culture-based method as *Aspergillus fumigatus* were sub-cultured on Sabouraud dextrose agar plate at 37°C for 7 days. Species identification will be confirmed by DNA sequencing.

Genomic DNA extraction

For genomic DNA extraction, *Aspergillus fumigatus* clinical isolates were sub-cultured on Sabouraud dextrose agar plate at 37°C for 3 days. After that the fungal conidia were cultured and shaken in glucose minimum media broth (GMM broth) at 37°C and 250 rpm for overnight. Then, after centrifugation at 4500 rpm for 15 min at room temperature, 600 µl TENS buffer and sterile glass beads were added to the cell pellet and mixed by homogenizer. The cell suspension was incubated at 65°C for 1 h prior to genomic DNA extraction using plant genomic DNA extraction kit following the manufacturer instructions (Viogene, Taiwan). The obtained DNA was quantified by UV-spectrophotometer.

DNA sequencing

DNA sequencing was performed to confirm species identification of the *Aspergillus fumigatus* isolates. For DNA sequencing, genomic DNA wasamplified by PCR, using the primer set ITS1 (5´-TCC GTA GGT GAA CCT GCG G-3´) and ITS4 (5´-TCC TCC GCT TAT TGA TAT GC-3´) which target the conserved internal spacer (ITS) region of the ribosomal DNA. Cycle parameters were 5 minutes at 95°C (initial denaturation), 35 cycles of 30 seconds at 95°C (denaturation), 1 minute at 55°C (annealing), and 1 minute at 72°C (extension) before 5 minutes at 72°C (final extension). Depending on species, the expected size of the amplicon was in the range of 300-500 bp. PCR products were checked on agarose gel electrophoresis, and purified using PCR fragment purification kit. Finally, the PCR products were send to a company for sequencing using the same primer set.

Mating type determination

For determination of mating type, genomic DNA was subjected to multiplex PCR as previously described by Paoletti et al., 2005. The multiplex PCR will contain *MAT1-1* specific primer AFM1 (5´-CCTTGACGCGATGGGGTGG-3´), *MAT1-2* specific primer, AFM2 (5´-CGCTCCTCATCAGAACAACTCG-3´), and a common primer, AFM3 (5´-CGGAAATCTGATGTCGCCACG-3´) that targets the flanking region of *MAT* locus (Figure 1). In cases of no result, alternative primer sets were used such as *MAT1-1*-specific (AF52 [5´-GGAGGATGCCGGTCTTGG-3´] and AF32 [5´-TGGAGGCCGTTGAACAGG-3´]) or *MAT1-2*-specific (AF51 [5´-CCTCCCCATCAATGTGACC-3´] and AF31 [5´-CTCGTCTTTCCACTGCTTCC-3´]). Finally, gel electrophoresis was performed to check the size of the amplicons with the expected sizes being 834 bp and 438 bp for the *MAT1-1* and *MAT1-2*, respectively.

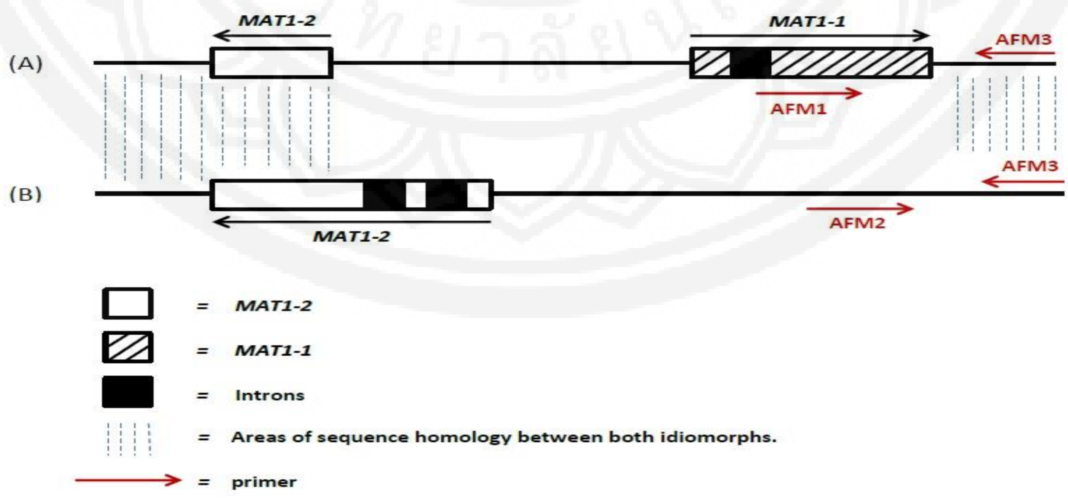


Figure 1 Diagram shows position of primers AFM1, AFM2 and AFM3, used for amplification of idiomorph regions.

Quantification of biofilm formation

Biofilm quantification was performed with slightly modification as described by Mowat et al. (Mowat, Butcher, Lang, Williams, & Ramage, 2007). Briefly, *Aspergillus fumigatus* clinical isolates were sub-cultured on Sabouraud dextrose agar plate at 37°C for 3 days. After that fungal conidia were harvested and diluted to 10^6 conidia/200 µl GMM broth. The diluted conidia were then inoculated into a 96 well culture plate and incubated at 37°C for 24h. The media was removed and the attached conidia were washed 3 times with 200 µl PBS. Biofilm formation were quantified by staining with 200 µl 0.5 % (w/v) crystal violet solution for 5 min, followed by removal of excess stain by keeping the plate under running water. Then, biofilm was destained with 200 µl of absolute ethanol and the destained suspension was subjected to absorbance reading at 590 nm. The experiment were done in triplicate and repeated twice with independent conidia preparations.

Results

Morphology of *A. fumigatus*

A. fumigatus of both mating types showed dark blue-green colonies on SDA plates (Figure 2a) with white to tan-colored reverse (Figure 2b). Microscopic morphology of *A. fumigatus* showed columnar conidial heads with uni-seriate conidia and smooth walled conidiophore (Figure 2c).

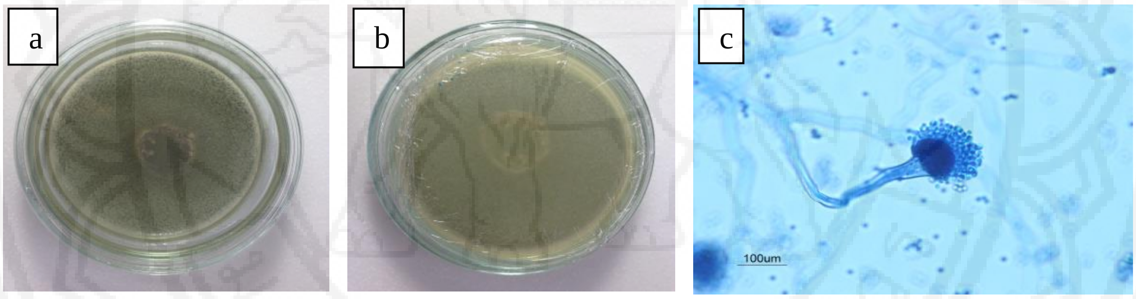


Figure 2 Macroscopic and microscopic examination of the *Aspergillus fumigatus*. (a = surface colony appearance on SDA, b = reverse colony appearance on SDA, c = conidiophore with conidia-covered vesicle)

Mating type determination

A single band of 834 bp was obtained for *MAT1-1* type and 438 bp was obtained for *MAT1-2* (Figure 3). Distribution of the mating type gene of the 23 isolates of *A. fumigatus* is shown in Table 1.

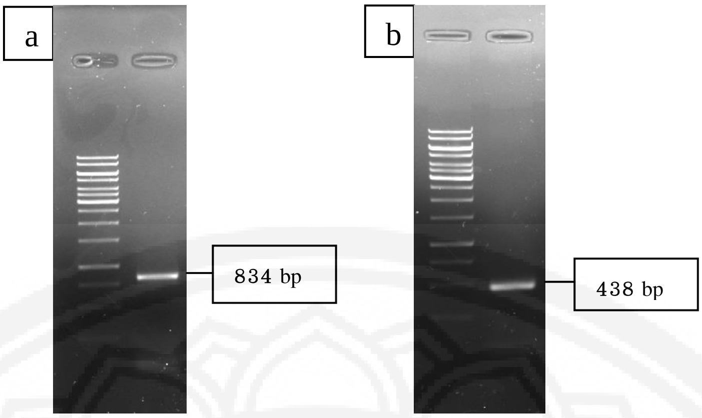


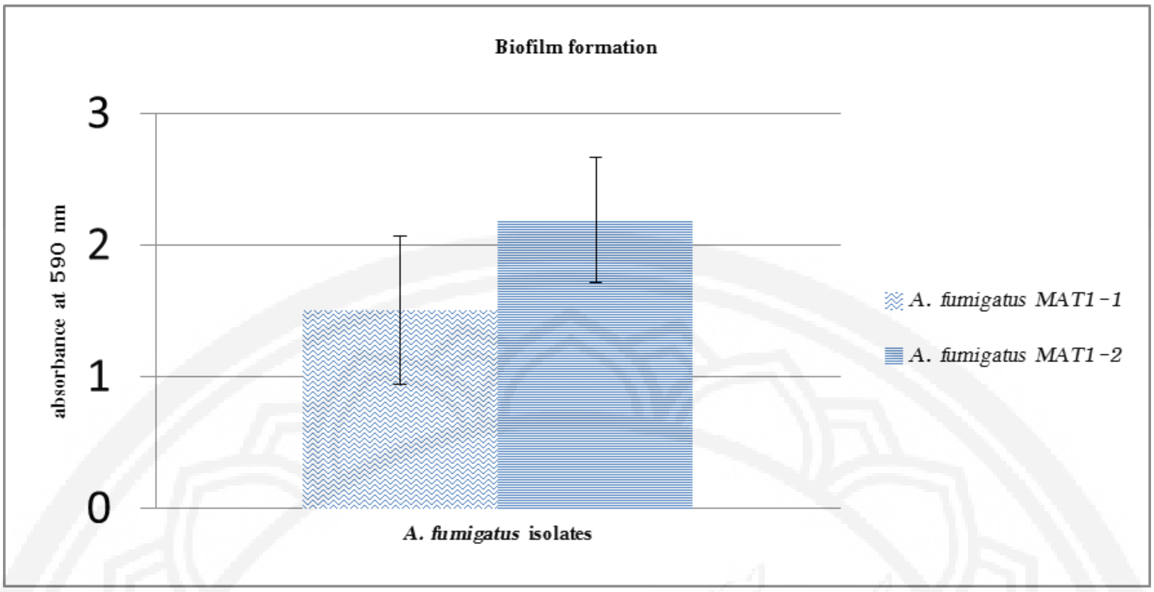
Figure 3 PCR product of mating type gene of *Aspergillus fumigatus*
3a = *MAT1-1*, 3b = *MAT1-2*.

Table 1 Specimen and mating type of *A. fumigatus* clinical isolates

Specimen type	**Mating type frequency**	
	MAT1-1	**MAT1-2**
Sputum	0	4
Bronchoalveolar lavage	1	2
Pus from abdominal wound	0	1
Pus from nasal cavity discharge	0	1
Pus from frontal bone	1	0
Pus from other site	0	1
Tissue biopsy from cervical lymph node	0	1
Tissue biopsy from mastoid	1	0
Tissue biopsy from right frontal bone	0	2
Tissue biopsy from right dural bone	0	2
Tissue biopsy from right temporal bone	1	0
Tissue biopsy from other site	0	1
aqueous humor	0	1
abdominal wound	1	0
lung effusion	0	1
mass at nasal cavity	0	1
Total	5	18

Quantification of biofilm formation

Biofilm formation of *A. fumigatus MAT1-1* and *MAT1-2* showed no statistically significant difference (p-value=0.491435) (Figure 4).



Figures 4 Biofilm formation of *A. fumigatus* of different mating type a = absorbance reading at 590 nm of 3 isolates of *A. fumigatus MAT1-1*. b = absorbance reading at 590 nm of 3 isolates of *A. fumigatus MAT1-2*.

Discussion

A. fumigatus is the most common cause of opportunistic fungal infection in immunocompromised host, especially in host with neutropenia and in bone marrow transplant recipients (Singh, 2005). Previously, *A. fumigatus* wasconsidered as asexual species, but recently, sexual cycle was discovered and sexual reproduction was successfully induced (O'Gorman et al., 2009). This finding raises the question whether it could have significant medical implications. In this study, we investigated relationship between mating type and biofilm formation as one of the virulence factor of *A. fumigatus*. As there are many steps involved in pathogenesis of *A. fumigatus*, basic knowledge of pathogenesis of this organism are important to prevent and treat infection in immunocompromised patients. Infection begins with inhalation of airborne conidia, which are widely distributed in the environment. Sometimes biofilm is formed after inhalation to evade from host immune response and antifungal drug. As relationship between mating type and virulence in *A. fumigatus* has not been much reported. In this study, we focused on the relationship between mating type and biofilm formation in *A. fumigatus*. The results revealed that *A. fumigatus* clinical isolates collected at Siriraj Hospital and Songklanagarind Hospital showed a higher proportion of *MAT1-2* isolates than *MAT1-1* isolates, in the ratio of about 3:1. This result correlated well with recent study that reported higher proportion of *MAT1-2* than *MAT1-1* in the ratio of about 2:1 (Bain et al., 2007). However, the result did not comply with those from Paoletti et al., 2005 who reported similar proportion between *MAT1-1* and *MAT1-2* isolates, both in environmental and clinical isolates. In addition, we found that both *MAT1-1* and *MAT1-2* were distributed in fungal isolates obtained from several organs such as lung, nasal cavity, lymph node, mastoid and wounds (Table 1). For biofilm formation, we found no significant difference between the different mating types (*p*-value=0.491435). As all isolates that we used in this study derived from clinical specimen, it is possible that ability of biofilm formation may be affected by antifungals. Another possibility is that, mating type gene is in fact not involved in biofilm formation, but may play role in other more prominent virulence factors.

Conclusion and Suggestion

To our knowledge, this is the first study about relationship between mating type and biofilm formation in *A. fumigatus*. The results suggested that there is likely no relationship between mating type gene and biofilm formation. As there are several virulence factors that make *A. fumigatus* a successful pathogenic fungi, future study about relationship between *MAT* gene and others virulence factors of *A. fumigatus* may lead to better understanding of pathogenesis of this organism.

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