Original Article

Kurume Medical Journal, 69, 185-193, 2022

Molecular Analysis for Potential Hospital-Acquired Infection Caused by Aspergillus Tubingensis Through the Environment

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Received 13 July 2022, accepted 14 November 2022
J-STAGE advance publication 16 January 2024

Edited by YOSHITOSHI OGURA

Summary: The identification of *Aspergillus* species has been performed mainly by morphological classification. In recent years, however, the revelation of the existence of cryptic species has required genetic analysis for accurate identification. The purpose of this study was to investigate five *Aspergillus* section *Nigri* strains isolated from a patient and the environment in a university hospital. Species identification by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry identified all five black *Aspergillus* strains as *Aspergillus niger*. However, calmodulin gene sequence analysis revealed that all five strains were cryptic species, four of which, including the clinical strain, were *Aspergillus tubingensis*. Hospital-acquired infection of the patient with the *A. tubingensis* strain introduced from the environment was suspected, but sequencing of six genes from four *A. tubingensis* strains revealed no environmental strain that completely matched the patient strain. The amount of in vitro biofilm formation of the four examples of the *A. tubingensis* strain was comparable to that of *Aspergillus fumigatus*. An extracellular matrix was observed by electron microscopy of the biofilm of the clinical strain. This study suggests that various types of biofilm-forming *A. tubingensis* exist in the hospital environment and that appropriate environmental management is required.

Keywords black *Aspergillus*, *Aspergillus* section *Nigri*, *Aspergillus tubingensis*, multilocus sequence analysis, biofilm

INTRODUCTION

Aspergillus species are ubiquitous in environmental elements such as soil, water, food, fireproof building materials, and ventilation equipment and cause both invasive and non-invasive aspergillosis infections in immunocompromised patients. The fungi produce a large number of conidia and extensively contaminate the environment [1]. Among *Aspergillus* species, *Aspergillus fumigatus* is the main causative agent of aspergillosis infections, such as pulmonary aspergilloma, invasive pulmonary aspergillosis, and otomycosis [2-4]. Other important causative agents of aspergillosis include *Aspergillus flavus*, *Aspergillus* niger, *As-*

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Abbreviations: DDBJ, DNA data bank of Japan; ECM, extracellular matrix; ICT, infection control team; ITS, internal transcribed spacer; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry; Mcm7, minichromosome maintenance factor 7; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PDM, potato dextrose medium; RPB2, RNA polymerase Ⅱ second largest subunit; SEM, scanning electron microscopy; Tsr1, ribosome biogenesis factor.

pergillus terreus, and *Aspergillus nidulans*. Conventional identification of *Aspergillus* species using microscopic and macroscopic morphological criteria in the clinical laboratory often misidentifies cryptic species. Accurate identification of *Aspergillus* species, including cryptic species, requires gene sequence analysis [5,6].

The *Aspergillus* section *Nigri* contains *A. niger*, which is the most recognized species of the section, along with cryptic species such as *Aspergillus aculeatus*, *Aspergillus carbonarius*, *Aspergillus japonicus*, *Aspergillus uvarum*, *Aspergillus tubingensis*, and *Aspergillus welwitschiae*. Many isolates of the cryptic species of *Aspergillus* section *Nigri* have been misidentified as *A. niger* [7,8]. In Japan, Species belonging to *Aspergillus* section *Nigri* are frequently isolated from clinical specimens next to *A. fumigatus*. [9,10]. The recently reported number of clinical isolates of *A. niger* is less than that of its cryptic species, especially *A. tubingensis* and *A. welwitschiae* [8]. *A. niger* and its cryptic species are known to have different drug susceptibilities. *A. tubingensis* is one of the major species isolated from patients and is known to be less susceptible to itraconazole and azole antifungal agents other than *A. niger* [11-14]. In this study, one strain in the *Aspergillus* section *Nigri* was isolated from the sputum of a patient with pulmonary aspergillosis hospitalized at Kurume University Hospital, and four strains of the *Aspergillus* section *Nigri* were isolated from the hospital locker room. The purpose of this study was to investigate the possibility of hospital-acquired infection of the environmental strain.

MATERIALS AND METHODS

Ethical Approval: All studies described herein were approved by the Human Ethics Review Boards of Kurume University (Research No. 21030).

Case: On September 1st, 2019, a 48-year-old female was rushed to a municipal hospital for an emergency that was due to an exacerbation of respiratory distress during the night. She had been undergoing steroid treatment for overlap syndrome since 2012. The patient went into cardiopulmonary arrest upon arrival at the hospital. Her heartbeat was resumed with cardiopulmonary resuscitation, and she was then transferred to Kurume University Hospital for suspected cardiopulmonary arrest due to acute adrenal insufficiency.

On hospital admission, pulmonary hypertension was observed, and the β-D-glucan value was below the detection limit (< 6.0 pg/mL) of the Wako β-D-glucan test (FUJIFILM Wako Pure Chemical Industries, Tokyo, Japan). A blood test on the 9th day of hospitalization showed that the β-D-glucan level had increased to 23.7 pg/mL with a cut-off of 11 pg/mL. On the 12th day, the *Aspergillus* antigen value of the Platelia *Aspergillus* antigen enzyme immunoassay (Bio-Rad Laboratories, Hercules, CA, USA) was 0.7 with a cut-off of 0.5, and the β-D-glucan value was 36.0 pg/mL, and a chest X-ray image showed deterioration. Simultaneously, A sputum culture test showed no growth of respiratory pathogens other than black *Aspergillus*.

The patient was treated with liposomal amphotericin B and caspofungin as antifungal agents for use in patients with pulmonary aspergillosis with impaired liver function. After that, the patient progressed well and was transferred to the general ward on the 24th day. On the 41st day, the β-D-glucan level tended to decrease, antifungal medication was discontinued, and the patient was discharged in remission.

Black *Aspergillus* isolates: Black *Aspergillus* strain P was isolated from the sputum of the above-mentioned patient on September 14th, 2019. In the same month of the same year, a number of complaints about environmental contamination were received from staff using the locker room at Kurume University hospital, which prompted the infection control team (ICT) to intervene. On September 24th, the ICT conducted a check of the status and growth of many filamentous fungi that had been observed on the air conditioner grille, white coats, and surfaces of the lockers, so an environmental culture was carried out. Four environmental strains growing on the grilles of two air conditioners in the locker room were isolated by environmental testing; two were isolated on September 24th, 2019 (strains E1 and E2), and two on August 27th, 2020 (strains E3 and E4). The ward was cleaned daily, and no filamentous fungal growth was observed. Therefore, the ICT speculated that black *Aspergillus* breeding in the locker room could have been transmitted to the patient. A total of five strains were analyzed.

Species identification and multilocus sequence analysis: First, five black *Aspergillus* strains were identified by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) (Biotyper smart; Bruker Daltonics Inc., Billerica, MA, USA) using the database from the MBT Filamentous Fungi Library version 2.0. Samples were prepared according to the vendor's instructions, as described previously [15]. Next, calmodulin gene sequence analysis for species identification and multilocus sequence analysis for strain identity were per-

formed using polymerase chain reaction (PCR). The calmodulin gene is useful in discriminating species in *Aspergillus* section *Nigri* [16]. Several gene sequences are used for detailed species identification, including cryptic species [17,18]. Black *Aspergillus* strains were grown on potato dextrose agar (BD, Sparks, MD, USA) at 25°C for 5-7 days. A small amount of mycelium from each strain was retrieved using a toothpick. Genomic DNA from each sample was extracted using microLYSIS-PLUS (Gel Company, Inc., San Francisco, CA, USA) according to the manufacturer's protocol. PCR amplified several factors: calmodulin; internal transcribed spacer (ITS); β-tubulin; minichromosome maintenance factor 7 (Mcm7); RNA polymerase Ⅱ (RPB2), which was the second largest submit; and ribosome biogenesis factor (Tsr1) loci. The sets of PCR primers used are shown in Table 1. Primers for ITS [19] and β-tubulin [20] are commonly used primers, and the others were original primers. The analyzed DNA sequences were subjected to a BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi). DNA sequence data were deposited to the DNA Data Bank of Japan (DDBJ).

Quantitative biofilm assay: The biofilm formations of four *A. tubingensis* strains were compared using quantitative biofilm assay. To 1.5 mL potato dextrose medium (PDM), 1×10^6 CFU of conidia was added (BD, Sparks, MD, USA) using 24-well plates, and samples of the combination were incubated at 27 and 37°C for 24 h. The medium was changed, and the plates were further incubated for another 24 h. After rinsing with phosphate-buffered saline (PBS), 1 mL of PBS was added, and the biofilms were collected using cell scrapers (9000-220; AGC Techno Glass, Shizuoka, Japan), and these were then transferred to microtubes. The samples were centrifuged at 15,000 rpm for 5 min. The supernatants were removed, and the precipitates were treated with ethanol at 4°C for 5 min and centrifuged at 15,000 rpm for 5 min. The supernatants were removed, and the precipitates were dried in a heat block at 70°C for 5 h in a safety cabinet with a fan running. The dried materials were weighed using a semi-micro balance. This experiment was carried out seven times. *A. fumigatus* environmental isolates IMF 62630 and IMF 64772 and the clinical isolates IMF 65972 and IMF 65974 were used as controls.

Electron microscopy: Biofilm formation of strain P was assessed by Scanning electron microscopy (SEM). Biofilm formation was initiated on a glass piece with 1×10^6 CFU of conidia in 1.5 mL of PDM for each well of a 24-well plate, and the plate was incubated at 37°C for 24 h. The medium was changed, and the plate was further incubated for 24 h. After removing the PDM, the biofilms were rinsed with HEPES buffer consisting of 30 mM HEPES-KOH (pH7.4), 100 mM NaCl, and 2 mM $CaCl_2$ and fixed with HEPES buffered fixative containing 2.5% glutaraldehyde, 2% formaldehyde, and 2 mM $CaCl_2$ for 2 h. For SEM observation, the specimens were fixed with 1.5% potassium ferrocyanide-reduced 2% osmium tetroxide, rinsed with buffer, reacted with 1% thiocarbohydrazide for 30 min, and then further fixed with 2%

TABLE 1.
PCR primers used in this study

Primers	Target	Primer sequences (5' to 3')
CF1L- Nigri	Calmodulin gene	CTCTCTGACCGAAGAGCAAGTTTC
CF4- Nigri		TGCATCATGAGCTGGACGAACTC
ITS1	ITS	TCCGTAGGTGAACCTGCGG
ITS4		TCCTCCGCTTATTGATATGC
Bt2a	β-tubulin gene	GGTAACCAAATCGGTGCTGCTTTC
Bt2b		ACCCTCAGTGTAGTGACCCTTGGC
Mcm7F- Nigri	*Mcm7* gene	AACCCGTGTCTCGGATGTCAAG
Mcm7R- Nigri		TGGCAACACCAGGGTCACCCATG
RPB2F- Nigri	*RPB2* gene	CTCATGTGCTACATCACTGTTGG
RPB2R- Nigri		GAYTGGTTGTGGTCGGGGAAAGG
Tsr1F- Nigri	*Tsr1* gene	ATGAGCGCAGCTGGATGTTC
Tsr1R- Nigri		AACTCCAAATCTTCGTTGGCCT

osmium tetroxide. After rinsing with double distilled water, the specimens were dehydrated in a graded ethanol series, substituted *t*-butyl alcohol, and freeze-dried under vacuum. The dried samples received an osmium conductive metal coating and were observed via SEM (JSM-IT800; JEOL, Japan).

RESULTS

Isolation of black *Aspergillus* strains: In September 2019, the inpatient's respiratory status had exacerbated (Fig. 1), and a clinical strain (strain P) was isolated from the patient's sputum. The laboratory tests showed an elevation of white blood cell count and c-reactive protein (Table 2), which might have

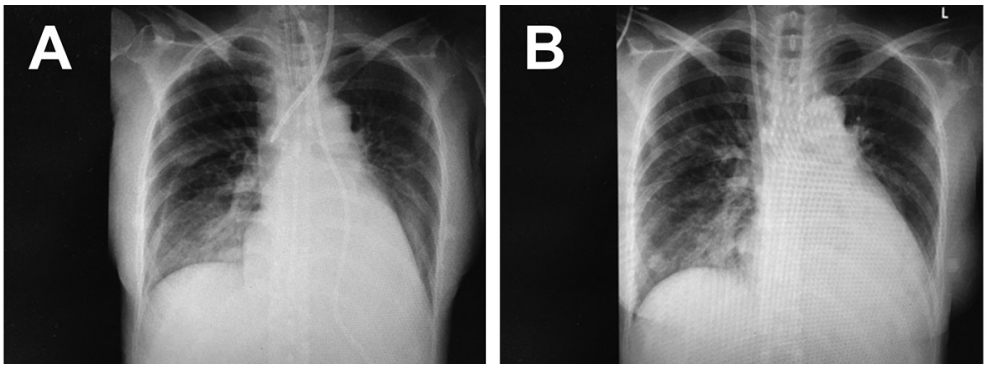

Fig. 1. Chest radiographs.
(A) Day 5. Hazy opacities mainly in the right and left lower lung zones.
(B) Day 12. Exacerbation in the middle and lower lobes of the right lung.

TABLE 2.
Laboratory tests of the patient during hospitalization

Test	Normal values	1st day	12th day
Chemistry and Hematology			
CRP (mg/dL)	< 0.14	0.84	5.64
AST (U/L)	13-30	687	27
ALT (U/L)	7-30	348	56
LDH (U/L)	124-222	1663	326
ALB (g/dL)	4.1-5.1	2.7	2.5
WBC (10^3/μL)	3.3-8.6	11.7	32.6
Defferential (%)			
Neutrophil	40-71.9	62.6	93.1
Lymphocyte	26.0-46.6	32.7	5.1
PLT (10^3/μL)	158-348	337	114
Coagulation			
PT (seconds)	11-13	20.9	12.1
INR	0.85-1.15	1.60	1.00
Biomarker			
PCT (ng/mL)	< 0.5	0.03	13.76
β-D-glucan (pg/mL)	< 11.0	< 6	36.0

CRP, c-reactive protein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; ALB, albmin; WBC, white blood cell; PLT, platelet; PT, prothrombin time; INR, international normalized rtio; PCT, procalcitonin

been caused by increased steroid administration during inpatient treatment.

Simultaneously, black *Aspergillus* was found growing in the locker room at the university hospital. The ICT observed fungal contamination during environmental testing. Fabric laundry boxes for white coats, white coats on hangers, and the external and internal surfaces of lockers were contaminated (Fig. 2). Specimens collected from contamination were cultured, and a number of filamentous fungi were detected, which included black *Aspergillus* strains E1 and E2. Subsequently, the locker room was cleaned. Ventilation fans, which had been out of operation, were activated. The room was cleaned regularly, several dehumidifiers were installed, and the temperature and humidity of the room were monitored. However, fungal contamination was again observed during the next environmental testing performed in August 2020, and filamentous fungi of black *Aspergillus* strains E3 and E4 were detected.

MALDI-TOF MS and multilocus sequence analysis of black *Aspergillus* strains: Using MALDI-TOF MS at the species level, five Black *Aspergillus* strains were identified as *A. niger* (score value > 2.0). However, these score thresholds have been designed for bacterial species identification and are not necessarily appropriate for filamentous fungi [21]. Calmodulin gene partial sequence analysis of *A. niger* revealed the strain P and environmental isolates (strains E2, E3, and E4) to be *A. tubingensis*, and strain E1 was *A. welwitschiae*. All strains are cryptic species of *A. niger*. Because the reference database used for MALDI-TOF MS did not contain the reference spectra of *A. tubingensis* and *A. welwitschiae*, these strains were misidentified as *A. niger* using MALDI-TOF MS. Four *A. tubingensis* strains were further analyzed by multilocus sequence analysis (Table 3). At the ITS and RPB2 loci, all four strains showed the same sequence. The sequence of the calmodulin gene of strain P was the same as that of strain E4, and two base substitutions compared to strains E2 and E3. The sequence of the β-tubulin gene of strain P was the same as those of strains E3 and E4, and one base substitution and two single-base deletions in intron 3 compared to that of strain E2. The sequence of the Mcm7 gene of strain P was the same as that of strain E3, with one base substitution compared to that of strain E2, and six base substitutions compared to that of strain E4. The sequence of the Tsr1 gene of strain P was the same as those of strains E2 and E3, while there were eight base substitutions compared to that of strain E4. These results showed that the four *A. tubingensis* strains were different from one another. In ITS and RPB2, sequences were identical in the four strains.

Biofilm formation: Samples of the conidia of the four *A. tubingensis* strains were cultured separately in 24-well plates at 27 and 37°C for 48 h. Four strains of *A. fumigatus*, the primary causative agent of aspergillosis, were used as controls. The dry weights of the biofilms were measured (Fig. 3). The *A. fumigatus* strains tended to produce more biofilm at 27°C than at 37°C. On the other hand, there was no significant difference in the amount of biofilm produced by *A. tubingensis* between 27 and 37°C. The amount of biofilm produced by *A. tubingensis* was comparable to that of *A. fumigatus*. This suggests that *A. tubingensis* is capable of forming biofilms under hospital room temperature conditions as well as under normal human body temperature conditions.

Electron microscopy: The biofilm of *A. tubingensis* strain P was observed using SEM. The extracellular matrix (ECM) was not developed throughout the mycelium, but a hump-shaped, long ribbon-shaped, or plate-shaped ECM was observed in some places (Fig. 4).

DISCUSSION

In recent years, hospital-acquired fungal infections have increased worldwide. For immunocompromised patients and the elderly, invasive fungal infections caused by filamentous fungi are associated with high rates of morbidity and mortality, and appropriate treatment must be initiated as soon as possible. The common species *A. fumigatus*, *A. niger*, *A. flavus*, and *A. terreus* have been identified by morphological observations. However, with the development of DNA sequencing analysis, it has become clear that there are many cryptic species of *Aspergillus* that are morphologically difficult to distinguish from common species [22,23]. Invasive aspergillosis due to cryptic *Aspergillus* species has been reported, and accurate identification of the species is required.

A. niger is a representative species of the *Aspergillus* section *Nigri*. Its cryptic species, such as *A. tubingensis* and *A. welwitschiae*, are very likely to be misidentified as *A. niger* by conventional morphological classification and MALDI-TOF MS methods [15,24,25]. DNA sequencing analysis has shown that a majority of clinical isolates previously identified as *A. niger* were actually either *A. tubingensis* or *A. welwitschiae* [26-29]. In this study, five black *Aspergillus* strains were initially identified as *A. niger* by MALDI-TOF MS, but this was changed when multilocus se-

Fig. 2. Environmental assessment by ICT.
The air conditioner grille (A), white coats (B), and surfaces of the lockers (C) were contaminated with filamentous fungi.

TABLE 3.
Strains, species, and DDBJ accession numbers

Strain	Species	calmodulin	ITS	β-tubulin	Mcm7	RPB2	Tsr1
P	*A. tubingensis*	LC707860	LC707861	LC707862	LC707863	LC707864	LC707865
E1	*A. welwitschiae*	LC707866	ND	ND	ND	ND	ND
E2	*A. tubingensis*	LC707867	LC707868	LC707869	LC707870	LC707871	LC707872
E3	*A. tubingensis*	LC707873	LC707874	LC707875	LC707876	LC707877	LC707878
E4	*A. tubingensis*	LC707879	LC707880	LC707881	LC707882	LC707883	LC707884

ND, not determined.

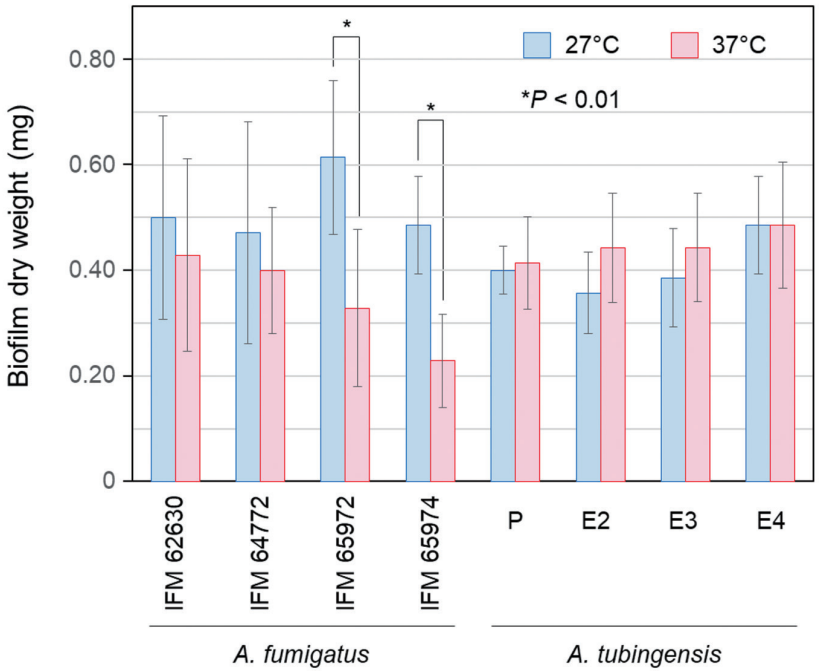

Fig. 3. Comparison of biofilm production of *A. fumigatus* and *A. tubingensis* strains.
Biofilm formation was performed in potato dextrose medium for 48 hours at 27°C and 37°C. Biofilms were collected and dried using a heat block at 70°C for 5 hours. The dry weight of the biofilm was measured.

quence analysis subsequently confirmed them to be cryptic species. The library provided by the vendor of the MALDI-TOF MS used in this study covers 152 species (or species groups) of filamentous fungi, of which only 28 belong to the genus *Aspergillus*, but the reference data for *A. tubingensis* and *A. welwitschiae* are not included. Accurate identification of black *Aspergillus* species by MALDI-TOF MS requires the addition of reference data for many cryptic species without omission [30]. Gene sequence analysis is highly reliable for the accurate identification of *Aspergillus* species, including cryptic species at present.

The four *A. tubingensis* strains, P, E2, E3, and E4, had identical sequences in two to five of the six genes analyzed. However, no environmental strain was identical to the P strain, and the route of infection could not be determined. There might have been many other *A. tubingensis* strains present in the hospital locker room where the four *A. tubingensis* strains were isolated in this study, and it is possible that some of these strains were the same as strain P. Therefore, we could not rule out the possibility that the P strain grew in the locker room and was introduced into the hospital through staff.

The most common route for filamentous fungi to enter a host is the inhalation of conidia. Therefore, it is necessary to maintain good air quality in critical areas of a hospital to reduce the incidence of invasive fungal infections [31]. In addition, Onami et al. reported that *Aspergillus* section *Nigri* strains were isolated from foods imported from overseas. Contamination of many food items suggests that respiratory and gastrointestinal colonization should be considered. Although rare, this could be a possible route of infection [32].

Biofilm production by microorganisms is known to be closely related to pathogenicity. Bacteria that form biofilms would be less susceptible to antimicrobial agents [33-35]. The fungus genus *Aspergillus* also produces biofilms, with *A. fumigatus* being the most prominent biofilm producer [36,37]. The general composition of bacterial and *Aspergillus* biofilms is polysaccharides, proteins, and nucleic acids, but there is a wide variety of detailed components among the species [38,39]. It has been reported that susceptibility to antifungal agents is reduced in biofilm-forming *Aspergillus fumigatus* [40]. The majority of *Aspergillus* section *Nigri* strains isolated from patients and the environment produce biofilms [41]. The *A. tubingensis* P strain isolated from one patient in this study and three strains isolated from the environment produced amounts of biofilm that were comparable to that of *A. fumigatus*, which is the preeminent pathogen of aspergillosis. As shown in Figure 2, *A. tubingensis* is thought to produce a biofilm that is pathologically equal to, or even higher than, that of *A. fumigatus* not only in the environment but also in the human body;

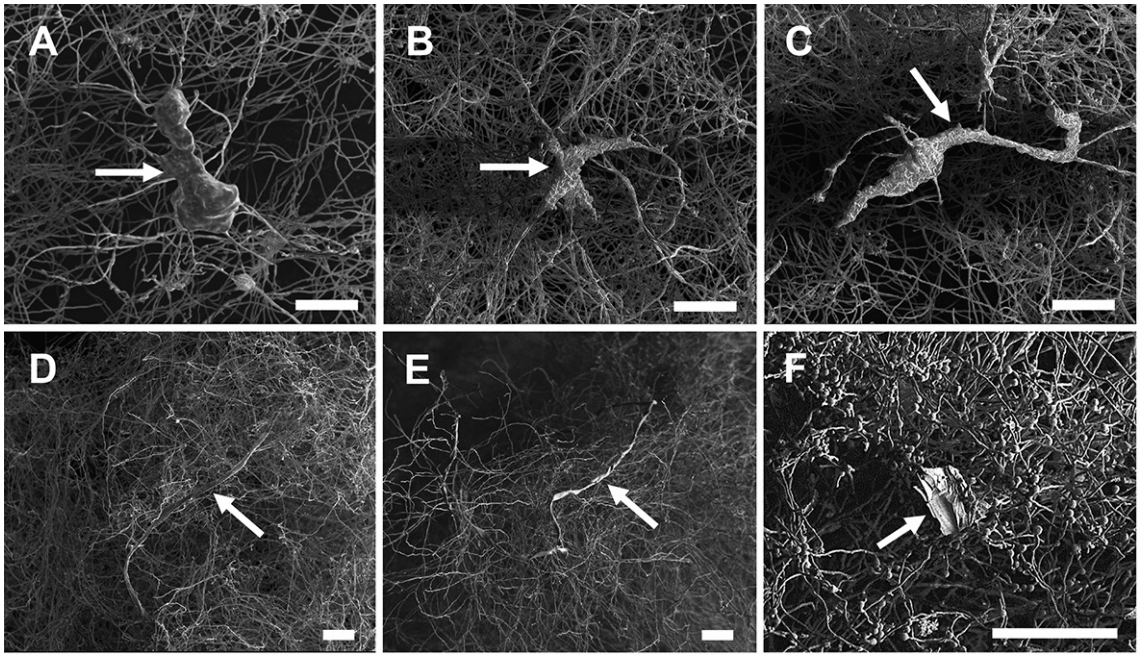

Fig. 4. SEM images of the biofilm of strain P.
The top (A-D) and bottom (E and F) surfaces of the biofilm. Hump-shaped ECM (A-C), long ribbon-shaped ECM along the hypha (D and E), and plate-shaped ECM (F) are indicated by arrows. Bars, 100 μm.

therefore, these two species are equally important to detect.

SEM observation of the biofilm of strain P showed ECM clumps in the form of humps, ribbons, and plates around the hyphae on the surface and bottom of the biofilm of strain P. It formed an ECM as bacteria and *A. fumigatus*, but the area where the developed ECM was observed was very limited. The significance of the morphological characteristics of the biofilm is unknown; biofilm formation might also contribute to enhanced virulence and decreased drug susceptibility of *A. tubingensis*. Therefore, environmental management is important to prevent hospital-acquired infection of *A. tubingensis*.

FUNDING: This study received no specific grant funding agents from the public, commercial, or not-for-profit sectors.

ACKNOWLEDGMENTS: We thank Hideki Katayama, Division of Infection Control and Prevention, Kurume University School of Medicine, for his assistance in this study.

CONFLICTS OF INTEREST: The authors declare no conflicts of interest.

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