



Original Article

Species identification, antifungal susceptibility, and clinical feature association of *Aspergillus* section *Nigri* isolates from the lower respiratory tract

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Abstract

Species of *Aspergillus* section *Nigri* are generally identified by molecular genetics approaches, whereas in clinical practice, they are classified as *A. niger* by their morphological characteristics. This study aimed to investigate whether the species of *Aspergillus* section *Nigri* isolated from the respiratory tract vary depending on clinical diagnosis. Forty-four *Aspergillus* section *Nigri* isolates isolated from the lower respiratory tracts of 43 patients were collected from February 2012 to January 2017 at the National Hospital Organization (NHO) Tokyo National Hospital. Species identification was carried out based on β -tubulin gene analysis. Drug susceptibility tests were performed according to the Clinical and Laboratory Standards Institute (CLSI) M38 3rd edition, and the clinical characteristics were retrospectively reviewed. *A. welwitschiae* was isolated most frequently, followed by *A. tubingensis*. More than half of the *A. tubingensis* isolates exhibited low susceptibility to azoles in contrast to only one *A. welwitschiae* isolate. Approximately three quarters of the patients from whom *A. welwitschiae* was isolated were diagnosed with colonization, whereas more than half the patients from whom *A. tubingensis* was isolated were diagnosed with chronic pulmonary aspergillosis (CPA). More attention needs to be given to the drug choice for patients with CPA with *Aspergillus* section *Nigri* infection because *A. tubingensis*, which was found to be frequently azole-resistant, was the most prevalent in these patients.

Key words: *Aspergillus* section *Nigri*, pulmonary aspergillosis, colonization.

Introduction

Pulmonary aspergillosis has several clinical manifestations and can cause life threatening conditions. Over 3.0 million and 4.8 million patients globally were diagnosed with chronic

pulmonary aspergillosis (CPA) and allergic bronchopulmonary aspergillosis (ABPA), respectively.^{1,2} The black aspergilli, that is, *Aspergillus* section *Nigri*, are the second most prevalent causative species of aspergillosis in humans, following *A.*

fumigatus, in Japan.^{3,4} Species causing aspergillosis differ across the globe. Species of *Aspergillus* section *Nigri* include *A. niger* (*sensu stricto*), *A. tubingensis*, *A. welwitschiae*, *A. uvarum*, and *A. brasiliensis*. Molecular genetics approaches, for example, DNA sequencing of calmodulin and β -tubulin genes, are performed to identify the species of this section.^{5–7} Several reports on the antifungal susceptibility of *Aspergillus* section *Nigri* have indicated that minimum inhibitory concentrations (MICs) of azoles, which are recommended as one of the first antifungal drugs to treat aspergillosis, were higher in *A. tubingensis* than the other species of *Aspergillus* section *Nigri*.^{6,8} In clinical practice, morphological identification of the isolated colonies fails to discriminate between the species of *Aspergillus* section *Nigri*,^{7,9} and they are all reported as *A. niger* (*sensu lato*), although some species, for example, *A. tubingensis*, exhibit a different phenotype microbiologically.

More attention should be given to the treatment of Pulmonary aspergillosis caused by *Aspergillus* section *Nigri* species, that is, *A. tubingensis*, which has low susceptibility to azoles. Therefore, the aim of this study was to investigate whether the species of *Aspergillus* section *Nigri* from the respiratory tract differ depending on clinical diagnosis—we examined which species of *Aspergillus* section *Nigri* were most prevalent in patients with CPA, ABPA, and colonization.

Methods

Aspergillus section *Nigri* isolates

Forty-four *Aspergillus* section *Nigri* isolates from 43 patients were collected from February 2012 to January 2017 at the NHO Tokyo National Hospital, Tokyo, Japan. Clinical samples, that is, sputum, bronchoalveolar lavage, endotracheal aspirate, and surgical samples, from the lower respiratory tract were cultured in Sabouraud dextrose agar (KANTO KAGAKU, Tokyo, Japan) or CHROM agar *Candida*/potato dextrose agar (KANTO KAGAKU, Tokyo, Japan) at 35°C for the first 2 days, and then the plates were further incubated at 22 ± 2°C for up to a total of 14 days. Then, colonies that showed morphological features of *A. niger* (*sensu lato*) were collected and purified on Sabouraud dextrose agar.

Species identification

All the isolates were identified to the species level by DNA sequencing of a part of β -tubulin gene based on the previously described methods.⁶

Susceptibility testing against antifungal drugs

Susceptibility test was performed according to the Clinical and Laboratory Standards Institute (CLSI) M38 3rd edition with partial modifications using the dried plate for antifungal

susceptibility testing (Eiken Chemicals, Tokyo, Japan, catalogue number: 9DEF47) as described previously¹⁰ to determine the MICs of itraconazole (ITCZ), voriconazole (VRCZ), and amphotericin B (AMB), and minimum effective concentrations (MECs) of micafungin (MCFG). The epidemiological cutoff values (ECVs) were defined as follows: ITCZ 2 µg/ml VRCZ 2 µg/ml, and AMB 2 µg/ml, based on the recommendations for *A. niger*.^{11,12} No ECVs have been established for MCFG.

Patient characteristics

We retrospectively analyzed the clinical data of 43 patients including the age, sex, underlying lung diseases, clinical diagnosis, and azole antifungal agents used before isolation.

Clinical diagnosis

CPA was diagnosed based on these three guidelines: guidelines for the management of deep-seated mycosis 2014, 3rd edition,¹³ guidelines by the Infectious Diseases Society of America,¹⁴ and guidelines by the European Respiratory Society.¹⁵ Briefly, CPA was diagnosed based on consistent symptoms, such as a few months of chronic pulmonary symptoms and consistent thoracic imaging of cavitation, pleural thickening, pericavitary infiltrates, or a fungal ball, and imaging in addition to the isolation of *Aspergillus* spp.

ABPA was diagnosed based on the criteria established by Agarwal et al.¹⁶ Briefly, ABPA was diagnosed when patients with bronchial asthma (1) exhibited positive type-I *Aspergillus* skin test or elevated *A. fumigatus*-specific immunoglobulin E (IgE) levels in addition to total IgE >1,000 IU/ml, or (2) met at least two of the following three criteria: (i) presence of precipitating or IgG antibodies against *A. fumigatus* in serum, (ii) radiographical pulmonary opacities consistent with ABPA, and (iii) total eosinophil count >500 cells/µl in steroid-naïve patients.

Colonization was defined as the lack of radiological and clinical findings suggestive of invasive aspergillosis (IA), CPA, and ABPA³ without new pulmonary infiltrates and symptoms.

There were no cases diagnosed with IA in this study.

Statistical analysis

Fisher exact test was used to determine (i) whether *A. tubingensis* isolates which had MICs to azoles above ECVs were affected by clinical use of azoles before isolation and (ii) whether *A. tubingensis* or *A. welwitschiae* was isolated more frequently among the patients with CPA or colonization. A *P* value <.05 was considered significant. The statistical analyses were performed using GraphPad Prism version 7.02 for Windows (GraphPad Software, La Jolla, CA, USA).

Ethics

The Institutional Review Board of NHO Tokyo National Hospital (approval date: July 24, 2013; approval number: 130020) approved the retrospective study, and written informed consent was not required.

Results

Species identification

Among the total 44 isolations of *Aspergillus* section *Nigri*, *A. welwitschiae* was isolated most frequently ($n = 22$, 50%), *A. tubingensis* was the second most frequent ($n = 17$, 38.6%), followed by *A. niger* ($n = 4$, 9.1%) and *A. uvarum* ($n = 1$, 2.3%). The sequences have been submitted in the NCBI database (the accession numbers: MK854718–61). Two isolates of *A. niger* were detected from one patient.

Susceptibility testing against antifungal drugs

MICs of ITCZ and VRCZ were determined for the isolated species as shown in Fig. 1. MICs of ITCZ and VRCZ for *A. tubingensis* ($n = 17$) were above ECVs in 64.7% ($n = 11$) and 70.6% ($n = 12$) of the isolated species, respectively. There was no significant difference in MICs above ECVs whether isolates were obtained from the patients who had used azoles prior isolation; 11/12 of 17 isolates were obtained from patients with no prior clinical use of azoles (ITCZ $P = .33$, VRCZ $P = .60$). On the contrary, there was only one *A. welwitschiae* isolate (4.5%) showing MIC above the ECVs. No isolates exhibited MICs above ECVs to AMB, and MECs to MCFG in all the isolates were ≤ 0.015 mg/l (data not shown).

Clinical diagnosis

The characteristics of the patients are shown in Table 1. As for the underlying pulmonary diseases, the number of patients with nontuberculous mycobacterial pulmonary disease was the highest ($n = 14$, 32.6%) followed by prior pulmonary tuberculosis ($n = 9$, 20.9%). Ten patients (23.3%) had been treated with azoles before obtaining *Aspergillus* isolates.

Among 43 patients, 17, 2, and 24 were diagnosed with CPA, ABPA, and colonization, respectively. Figure 2 indicates the clinical diagnosis of CPA/colonization for each species of *Aspergillus* section *Nigri*. Among patients with CPA, *A. tubingensis* was isolated most frequently ($n = 10$, 58.8%), whereas among patients with colonization, *A. welwitschiae* was isolated most frequently ($n = 16$, 66.6%) ($P = .023$) with significant difference ($P = .023$).

Approximately three quarters of the patients ($n = 16$, 72.7%) with *A. welwitschiae* isolation ($n = 22$) were diagnosed with colonization. More than half ($n = 10$, 58.8%) of the patients with *A. tubingensis* isolation ($n = 17$) were diagnosed with CPA.

Discussion

In this study, we determined the species of *Aspergillus* section *Nigri* isolated from the lower respiratory tract in patients with CPA, ABPA, and colonization to establish whether the *Aspergillus* species isolated from the respiratory tract vary depending on clinical diagnosis in *Aspergillus* section *Nigri*. *A. welwitschiae*, the most frequently isolated species in patients with colonization, was mostly susceptible to all the antifungal drugs tested. *A. tubingensis*, the second most prevalent, was often isolated from patients with CPA and exhibited low susceptibility to azoles.

We found that *A. tubingensis* was much more frequently isolated from patients with infections, not from those with colonization. In this regard, *A. tubingensis* might be more pathogenic than other species of *Aspergillus* section *Nigri*, such as *A. welwitschiae*. On the contrary, isolation of *A. welwitschiae* does not always mean lung infection caused by *Aspergillus*. Vermeulen et al. reported that among patients with IA, *A. tubingensis* was the most prevalent causative species¹⁷ while Balajee et al. reported that *A. niger* was the most prevalent followed by *A. tubingensis*.¹⁸ Among the species of *Aspergillus* section *Nigri*, clinical presentation, colonization, or infection, seems to be dependent on the virulence of the species in addition to association with local epidemiology.

In clinical practice, the problem is that *Aspergillus* section *Nigri* are diagnosed phylogenetically in most of the clinical laboratories.¹⁹ Although this section contains *A. tubingensis*, many of the isolates had low susceptibility to azoles. It is important to distinguish between the species of *Aspergillus* section *Nigri* using diagnostic tools such as nucleic acid amplification test.^{20,21} Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry may be another good identification tool in the future.²²

This study revealed that MICs of azoles against *A. tubingensis* were higher than those against the other species of *Aspergillus* section *Nigri*, which is consistent with the previous reports.^{6,8} Interestingly, higher MICs were not influenced by whether the isolates were obtained from patients with the use of azoles prior to isolation. This result suggests that several subspecies of *A. tubingensis* acquired resistance to azoles in the environment or had an intrinsic resistance to azoles.⁶ None of the isolates showed high MICs to AMB and MECs to MCFG in this study, which are alternative antifungal drugs for pulmonary aspergillosis.

Patients with pulmonary aspergillosis caused by *A. tubingensis* might have poor clinical outcomes similar to those caused by azole-resistant *A. fumigatus*,²³ although there is no recommendation for ECVs against azoles for *A. tubingensis*. For the treatment of cases with *A. tubingensis* isolation, it might be necessary to choose a high dose of azoles,²⁰ combination of azoles and echinocandin, or L-AMB.²³

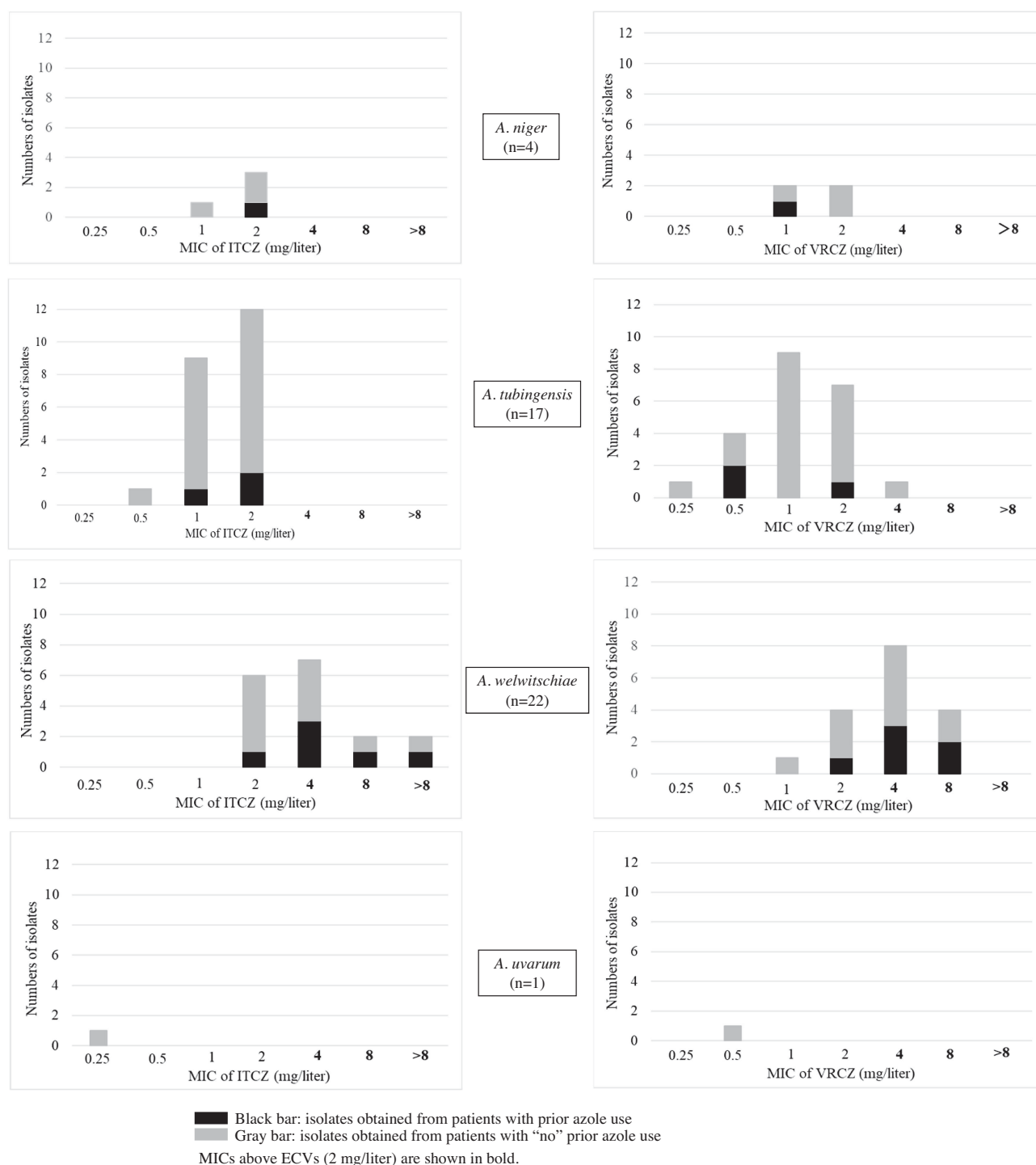


Figure 1. There were 11 (64.7%) and 12 *A. tubingensis* isolates (70.6%) revealing MICs of ITCZ and VRCZ above the ECVs, respectively whereas there was only one *A. welwitschiae* isolate (4.5%) showing MIC above the ECVs.

Limitations of this study were as follows: (1) isolates from a single center were analyzed, and the results might be influenced by local epidemiology.^{9,15} Multicenter analysis will be needed. (2) Pulmonary aspergillosis may develop in the patients with colonization, so these patients in this study should have been followed up.

In conclusion, *A. welwitschiae*, the most frequently isolated species of *Aspergillus* section *Nigri* from the lower respiratory tract, usually caused colonization, whereas *A. tubingensis*, the second most frequent and often resistant to azoles, caused CPA. More attention should be given to the drug choice for patients with CPA with *Aspergillus* section *Nigri* infection.

Table 1. Characteristics of patients ($n = 43$).

Age (years) ^a	69.6 ± 11.2
Male/Female ^b	22 (51.2)/ 21 (48.8)
Underlying pulmonary diseases ^{b,*}	
Nontuberculous mycobacterial pulmonary disease	14 (32.6)
Old pulmonary tuberculosis	9 (20.9)
Bronchial asthma	6 (14.0)
Interstitial lung disease	6 (14.0)
Chronic obstructive pulmonary disease	4 (9.3)
Bronchiectasis	4 (9.3)
History of thoracic surgery	1 (2.3)
Use of azole antifungal agents before isolation ^b	10 (23.3)
Duration of azole antifungal agents use before isolation ^{c,*}	
Itraconazole ($n = 9$)	363 (17–1594)
Voriconazole ($n = 5$)	132 (11–3650)

Date are presented as ^amean ± SD, ^b n (%), or ^cmedian (range).

*including cases of duplicate exposure.

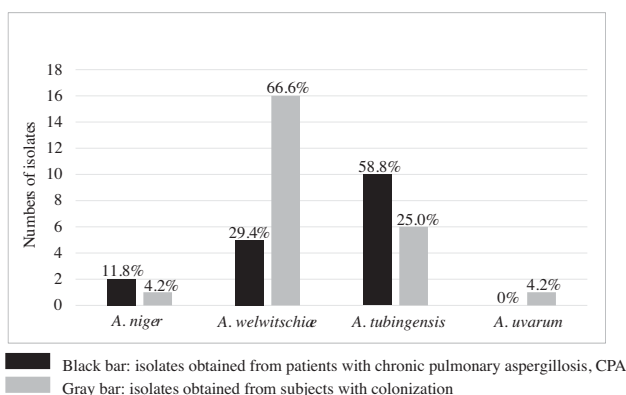


Figure 2. Among patients with CPA, *A. tubingensis* was isolated most frequently, whereas among patients with colonization, *A. welwitschiae* was isolated most frequently with significant difference ($p = 0.023$).

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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