# 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-

55 resistant, was the most prevalent in these patients.

56

57

58 **Introduction**

59 Pulmonary aspergillosis has several clinical manifestations and can cause life
60 threatening conditions. Over 3.0 million and 4.8 million patients globally were diagnosed
61 with chronic pulmonary aspergillosis (CPA) and allergic bronchopulmonary aspergillosis
62 (ABPA), respectively.[1,2] The black aspergilli, i.e. *Aspergillus* section *Nigri*, are the second
63 most prevalent causative species of aspergillosis in humans, following *A. fumigatus,* in
64 Japan.[3,4] Species causing aspergillosis differ across the globe. Species of *Aspergillus*
65 section *Nigri* include *A. niger* (*sensu stricto*), *A. tubingensis*, *A. welwitschiae*, *A. uvarum*,
66 and *A. brasiliensis*. Molecular genetics approaches, e.g. DNA sequencing of calmodulin
67 and β-tubulin genes, are performed to identify the species of this section.[5–7] Several
68 reports on the antifungal susceptibility of *Aspergillus* section *Nigri* have indicated that
69 minimum inhibitory concentrations (MICs) of azoles, which are recommended as one of
70 the first antifungal drugs to treat aspergillosis, were higher in *A. tubingensis* than the other
71 species of *Aspergillus* section *Nigri*.[6,8] In clinical practice, morphological identification
72 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, e.g. *A. tubingensis*, exhibit a different phenotype microbiologically.

More attention should be given to the treatment of Pulmonary aspergillosis caused by *Aspergillus* section *Nigri* species, i.e. *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.

## 2. Materials and Methods

### 2.1. *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, i.e. 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.

2.2. 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].

2.3. 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 previously[10] 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.

2.4. 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.

2.5. 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 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-naive patients.

127 Colonization was defined as the lack of radiological and clinical findings suggestive of
128 invasive aspergillosis (IA), CPA, and ABPA[3] without new pulmonary infiltrates and
129 symptoms.
130 There were no cases diagnosed with IA in this study.
131
132 2.6. Statistical Analysis
133 Fisher's exact test was used to determine i) whether *A. tubingensis* isolates which had
134 MICs to azoles above ECVs were affected by clinical use of azoles before isolation
135 and ii) whether *A. tubingensis* or *A. welwitschiae* was isolated more frequently among
136 the patients with CPA or colonization. A $p$ value $<0.05$ was considered significant. The
137 statistical analyses were performed using GraphPad Prism version 7.02 for Windows
138 (GraphPad Software, La Jolla California, USA).
139
140 2.7. Ethics
141 The Institutional Review Board of NHO Tokyo National Hospital (approval date: July
142 24th, 2013; approval number: 130020) approved the retrospective study and written
143 informed consent was not required.
144

145

146 **3. Results**

147 3.1. Species identification

148 Among the total 44 isolations of *Aspergillus* section *Nigri*, *A. welwitschiae* was

149 isolated most frequently (n=22, 50 %), *A. tubingensis* was the second most frequent

150 (n=17, 38.6 %), followed by *A. niger* (n=4, 9.1 %) and *A. uvarum* (n=1, 2.3 %). The

151 sequences have been submitted in the NCBI database (the accession numbers:

152 MK854718–61). Two isolates of *A. niger* were detected from one patient.

153

154 3.2. Susceptibility testing against antifungal drugs

155 MICs of ITCZ and VRCZ were determined for the isolated species as shown in Figure

156 1. MICs of ITCZ and VRCZ for *A. tubingensis* (n=17) were above ECVs in 64.7% (n=11)

157 and 70.6% (n=12) of the isolated species, respectively. There was no significant

158 difference in MICs above ECVs whether isolates were obtained from the patients who

159 had used azoles prior isolation; 11/12 of 17 isolates were obtained from patients with no

160 prior clinical use of azoles (ITCZ $p$=0.33, VRCZ $p$=0.60). On the contrary, there was only

161 one *A. welwitschiae* isolate (4.5%) showing MIC above the ECVs. No isolates exhibited

162 MICs above ECVs to AMB, and MECs to MCFG in all the isolates were ≤0.015 mg/liter

(data not shown).

### 3.3. 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%) ($p$=0.023) with significant difference ($p$=0.023).

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.

## 4. 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 species[17] 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.

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

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

213 Patients with pulmonary aspergillosis caused by *A. tubingensis* might have poor
214 clinical outcomes similar to those caused by azole-resistant *A. fumigatus*[23], although there
215 is no recommendation for ECVs against azoles for *A. tubingensis*. For the treatment of
216 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]

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.

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**Conflicts of interest**

None to declare.

## 5. References


1) Barac A, Kosmidis C, Alastruey-Izquierdo A, et al. Chronic pulmonary aspergillosis update: A year in review. *Med Mycol*. 2019; 57(Supplement_2): S104–S109.

2) Denning DW, Pleuvry A, Cole DC. Global burden of allergic bronchopulmonary aspergillosis with asthma and its complication chronic pulmonary aspergillosis in adults. *Med Mycol*. 2013; 51: 361–370.

3) Ohara S, Tazawa Y, Tanai C, et al. Clinical characteristics of patients with *Aspergillus* species isolation from respiratory samples: comparison of chronic pulmonary aspergillosis and colonization. *Respir Investig*. 2016; 54: 92–97.

4) Tashiro T, Izumikawa K, Tahiro M, et al. Diagnostic significance of *Aspergillus* species isolated from respiratory samples in an adult pneumology ward. *Med Mycol*. 2011; 49: 581–587.

5) Varga J, Frisvad JC, Kocsubé S, et al. New and revisited species in *Aspergillus* section *Nigri*. *Stud Mycol*. 2011; 69: 1–17.

6) Hashimoto A, Hagiwara D, Watanabe A, et al. Drug sensitivity and resistance mechanism in *Aspergillus* section *Nigri* strains from Japan. *Antimicrob Agents Chemother*. 2017; 61: e02583–e02616.

7) Samson RA, Noonim P, Meijer M, Houbraken J, Frisvad JC, Varga J. Diagnostic tools to identify black aspergilli. *Stud Mycol*. 2007; 59: 129–145.

8) Gautier M, Normand AC, L'Ollivier C, et al. *Aspergillus tubingensis*: a major filamentous fungus found in the airways of patients with lung disease. *Med Mycol*. 2016; 54: 459–470.

9) Abarca ML, Accensi F, Cano J, Cabañes FJ. Taxonomy and significance of black aspergilli. *Antonie Van Leeuwenhoek*. 2004; 86: 33–49.

10) Kikuchi K, Watanabe A, Ito J, et al. Antifungal susceptibility of *Aspergillus fumigatus* clinical isolates collected from various areas in Japan. *J Infect Chemother*. 2014; 20: 336-338.

11) Espinel-Ingroff A, Diekema DJ, Fothergill A, et al. Wild-type MIC distributions and epidemiological cutoff values for the triazoles and six *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). *J Clin Microbiol*. 2010; 48: 3251–3257.

12) Espinel-Ingroff A, Cuenca-Estrella M, Fothergill A, et al. Wild-type MIC distributions and epidemiological cutoff values for amphotericin B and *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). *Antimicrob Agents Chemother*. 2011; 55: 5150–5154.

13) Izumikawa K. Recent advances in chronic pulmonary aspergillosis. *Respir Investig*. 2016; 54: 85–91.

14) Patterson TF, Thompson GR 3rd, Denning DW, et al. Practice guidelines for the diagnosis and management of aspergillosis: 2016 update by the Infectious Diseases Society of America. *Clin Infect Dis*. 2016; 63: 433–442.

15) Denning DW, Cadranel J, Beigelman-Aubry C, et al. Chronic pulmonary aspergillosis: rationale and clinical guidelines for diagnosis and management. *Eur Respir J*. 2016; 47: 45–68.

16) Agarwal R, Chakrabarti A, Shah A, et al. Allergic bronchopulmonary aspergillosis: review of literature and proposal of new diagnostic and classification criteria. *Clin Exp Allergy*. 2013; 43: 850–873.

17) Vermeulen E, Maertens J, Meersseman P, Saegeman V, Dupont L, Lagrou K. Invasive *Aspergillus niger* complex infections in a Belgian tertiary care hospital. *Clin Microbiol Infect*. 2014; 20: O333–O335.

18) Balajee SA, Kano R, Baddley JW, et al. Molecular identification of *Aspergillus* species collected for the Transplant-Associated Infection Surveillance Network. *J Clin Microbiol*. 2009; 47: 3138–3141.

19) Frías-De-León MG, Rosas-de Paz E, Arenas R, et al. Identification of *Aspergillus*

*tubingensis* in a primary skin infection. *J Mycol Med*. 2018; 28: 274–278.

20) Palumbo JD, O'Keeffe TL. Detection and discrimination of four Aspergillus section *Nigri* species by PCR. *Lett Appl Microbiol*. 2015; 60: 188–195.

21) von Hertwig AM, Sant'Ana AS, Sartori D, et al. Real-time PCR-based method for rapid detection of *Aspergillus niger* and *Aspergillus welwitschiae* isolated from coffee. *J Microbiol Methods*. 2018; 148: 87–92.

22) D'hooge E, Becker P, Stubbe D, Normand AC, Piarroux R, Hendrickx M. Black aspergilli: A remaining challenge in fungal taxonomy? *Med Mycol*. 2018.doi: 10.1093/mmy/myy124. [Epub ahead of print].

23) Verweij PE, Ananda-Rajah M, Andes D, et al. International expert opinion on the management of infection caused by azole-resistant *Aspergillus fumigatus*. *Drug Resist Updat*. 2015; 21–22:30–40.

**Table 1. The characteristics of patients (n=43)**

| | |
|---|---|
| **Age (yrs)**[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 [a]mean±SD, [b]n (%), or [c]median (range)

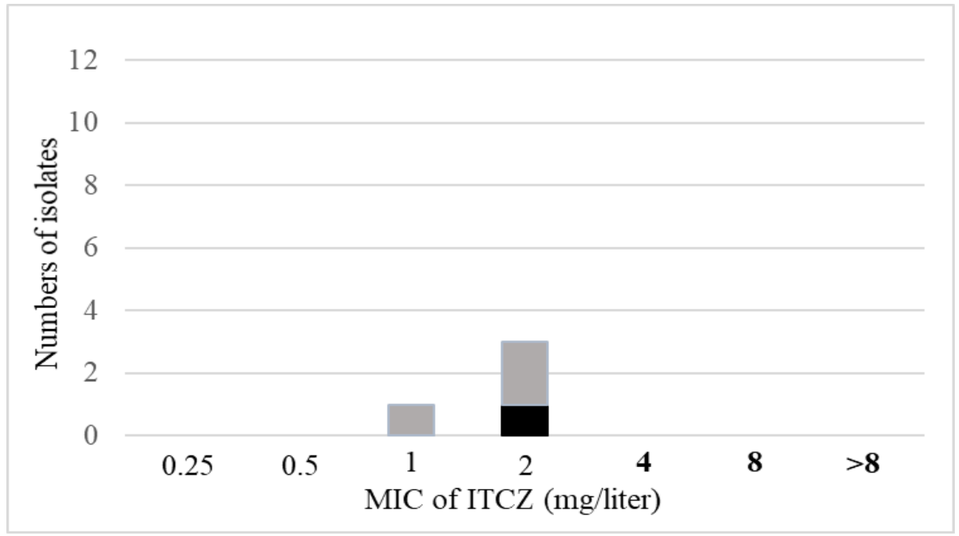
※including cases of duplicate exposure

**Figure Legends**

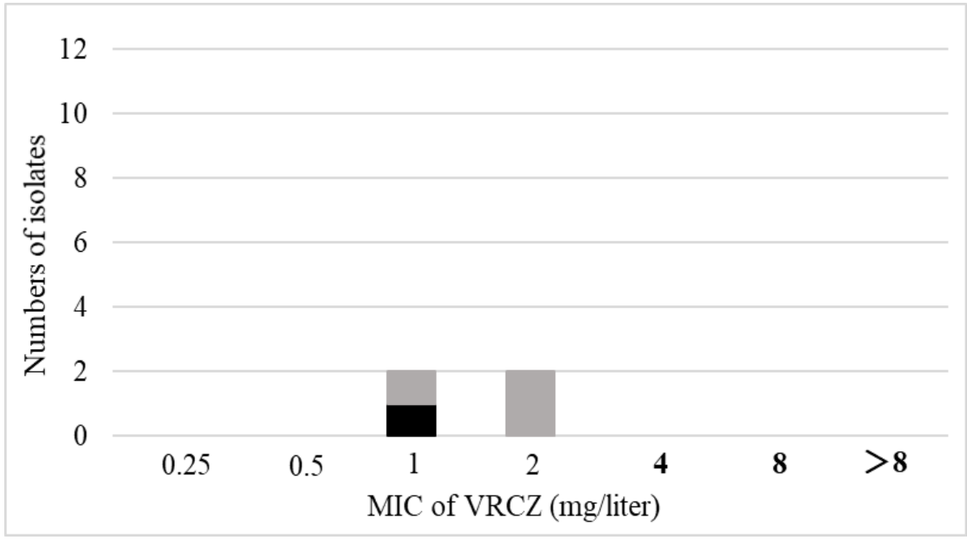
Fig. 1. There were 11 (64.7%) and 12 *A. tubingensis* isolates (70.6%) revealing MICs of ITCZ and VRCZ above the ECVs, respectively whereas there was only one *A. welwitschiae* isolate (4.5%) showing MIC above the ECVs.

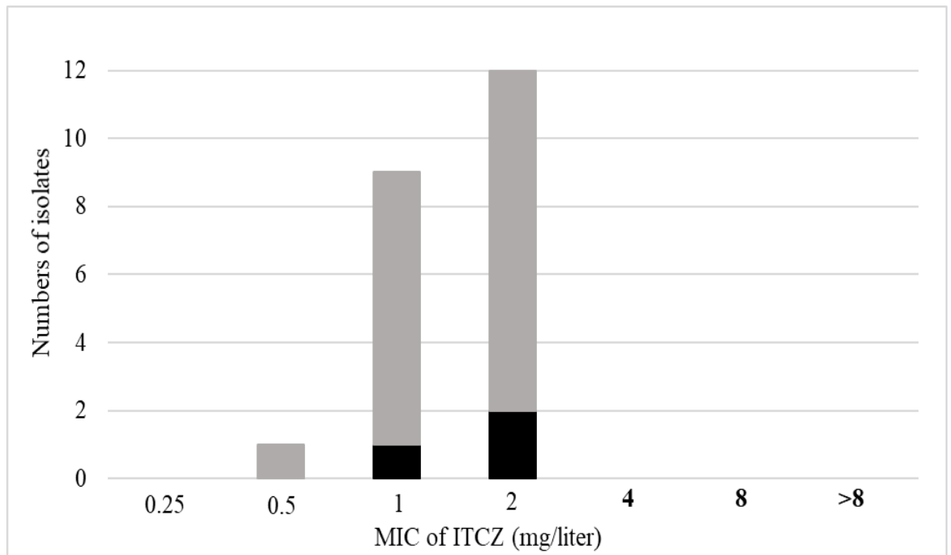
Fig. 2. Among patients with CPA, *A. tubingensis* was isolated most frequently, whereas among patients with colonization, *A. welwitschiae* was isolated most frequently with significant difference ($p$=0.023).

Figure 1. MICs of azoles for *Aspergillus* section *Nigri* isolates (n=44)

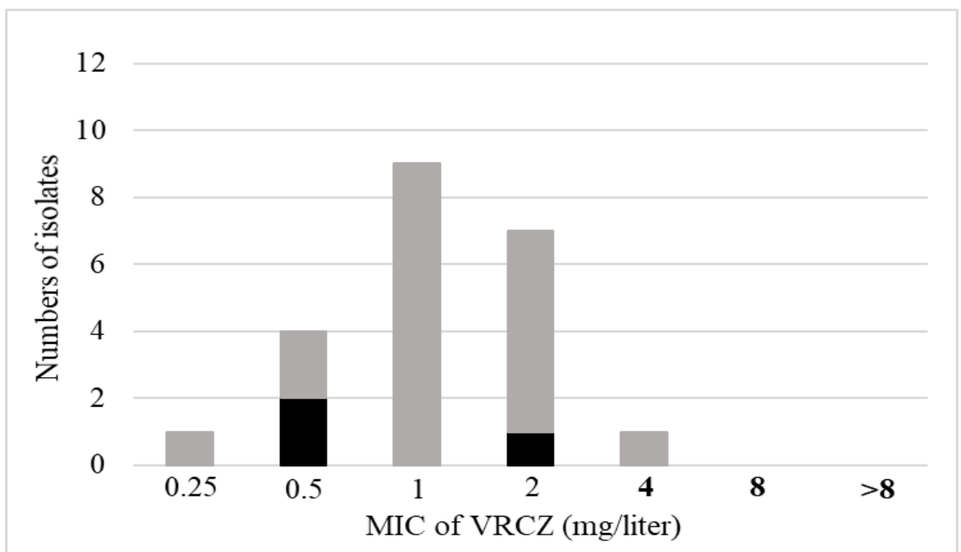


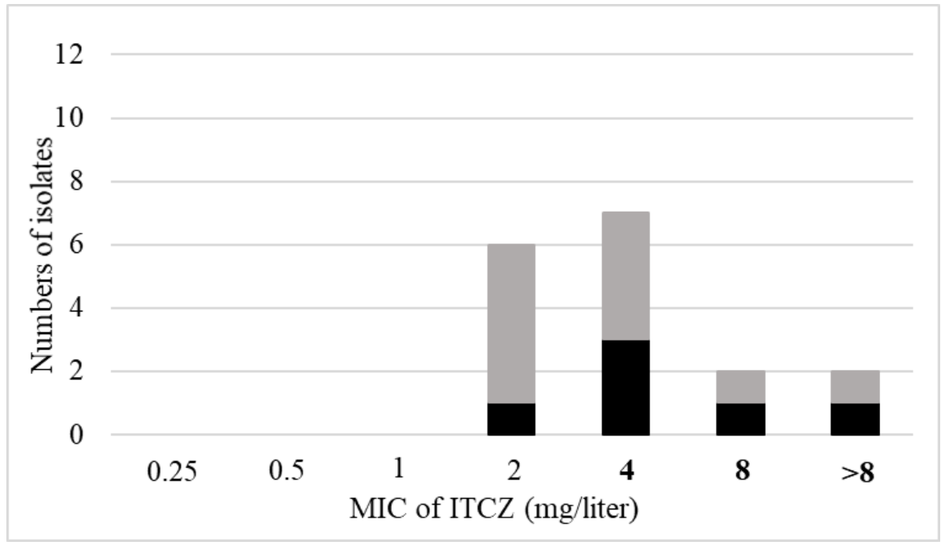

*A. niger* (n=4)



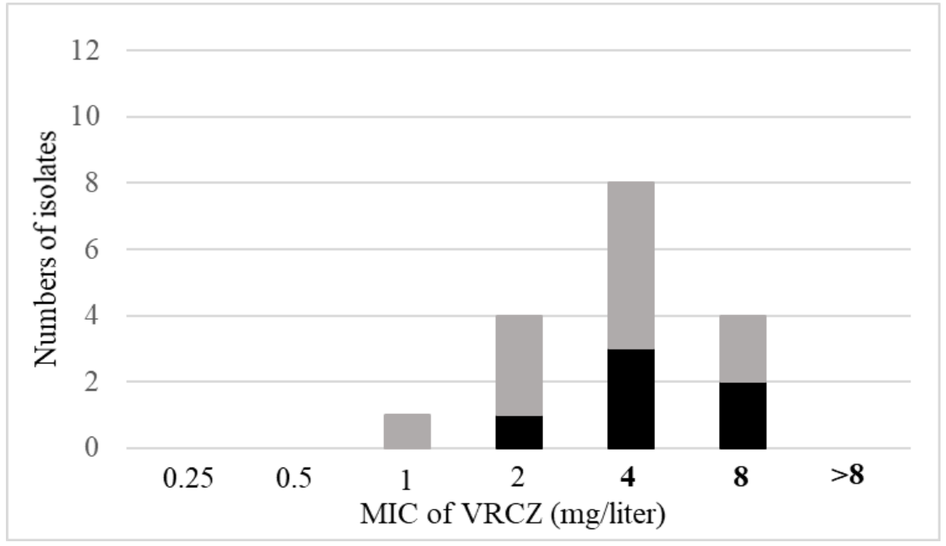



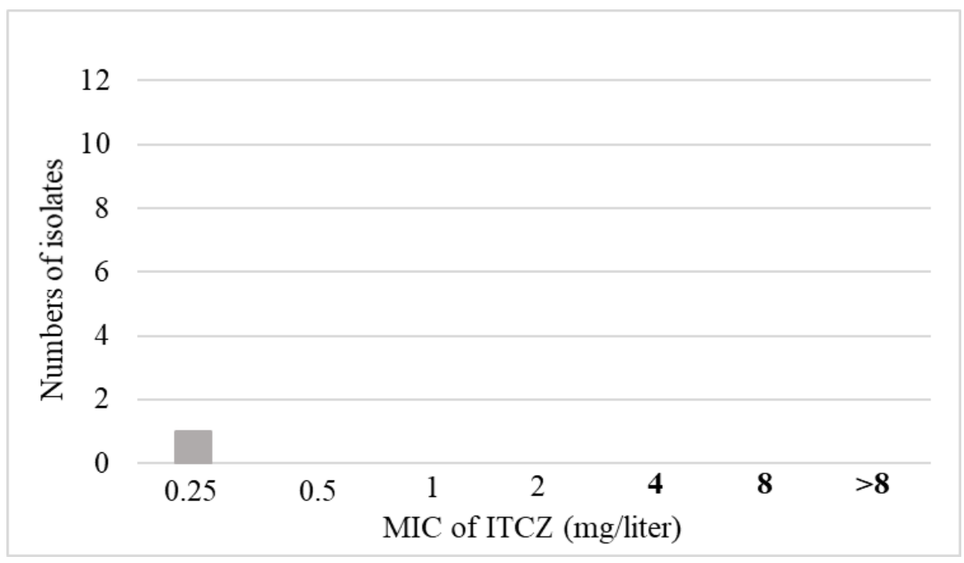

*A. welwitschiae* (n=22)



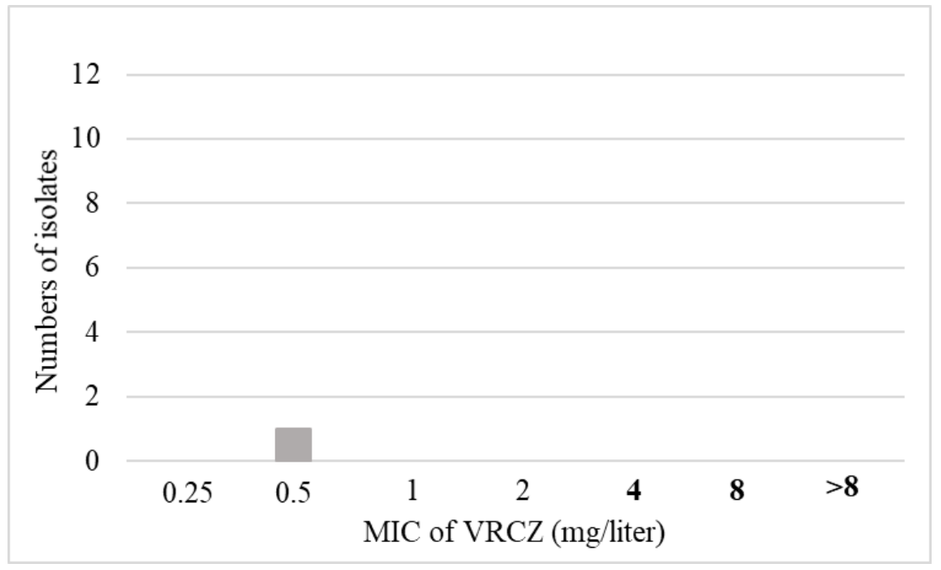



*A. tubingensis*
(n=17)




*A. uvarum*
(n=1)

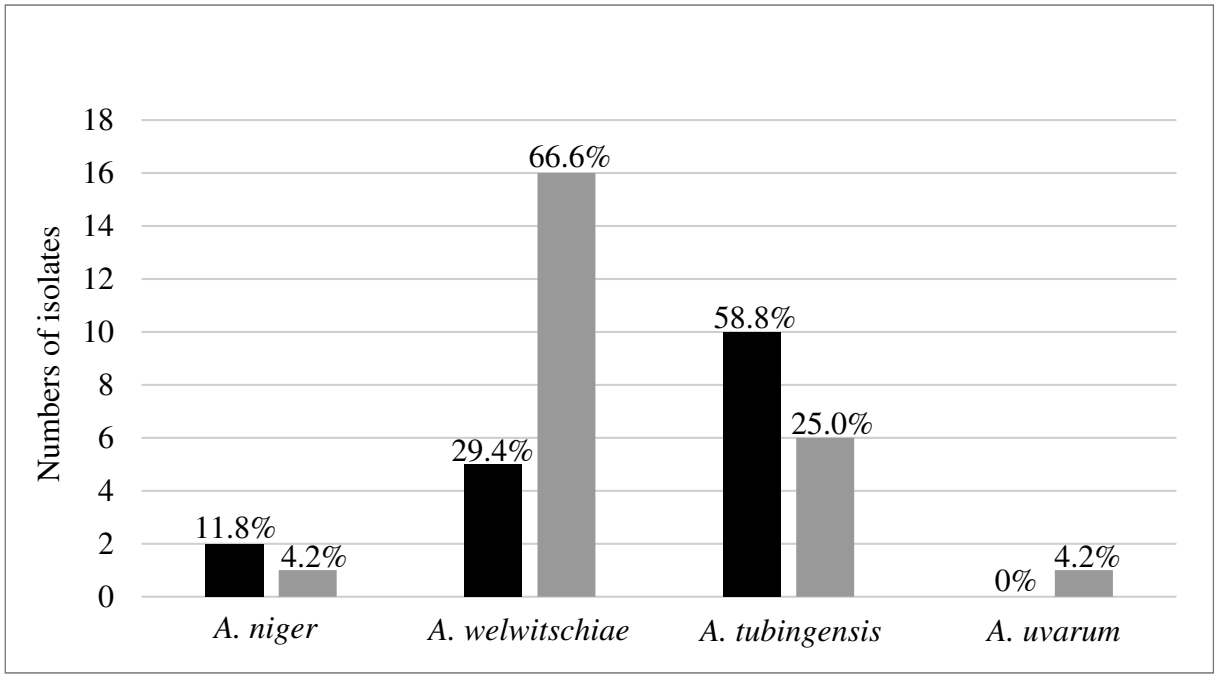



Black bar: isolates obtained from patients with prior azole use

Gray bar: isolates obtained from patients with “no” prior azole use

MICs above ECVs (2 mg/liter) are shown in bold.

Figure 2. Distribution of species among CPA (n=17) and colonization (n=24) groups.




Black bar: isolates obtained from patients with chronic pulmonary aspergillosis, CPA

Gray bar: isolates obtained from subjects with colonization