



Antifungal Susceptibility of the *Aspergillus viridinutans* Complex: Comparison of Two *In Vitro* Methods

Pavlina Lyskova,^{a,b} Vit Hubka,^{c,d} Lucie Svobodova,^a Vanessa Barrs,^e Navneet K. Dhand,^e Takashi Yaguchi,^f Tetsuhiro Matsuzawa,^g Yoshikazu Horie,^f Miroslav Kolarik,^{c,d} Radim Dobias,^{a,h} Petr Hamal^a

^aDepartment of Microbiology, Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic

^bLaboratory of Medical Mycology, Department of Parasitology, Mycology and Mycobacteriology Prague, Public Health Institute in Usti nad Labem, Prague, Czech Republic

^cDepartment of Botany, Faculty of Science, Charles University, Prague, Czech Republic

^dLaboratory of Fungal Genetics and Metabolism, Institute of Microbiology of the CAS, Prague, Czech Republic

^eSydney School of Veterinary Science and Marie Bashir Institute of Infectious Diseases and Biosecurity, University of Sydney, Camperdown, New South Wales, Australia

^fMedical Mycology Research Center, Chiba University, Chiba, Japan

^gDepartment of Nutrition Science, University of Nagasaki, Nagasaki, Japan

^hLaboratory of Clinical Mycology, Institute of Public Health, Ostrava, Czech Republic

ABSTRACT Cryptic species of *Aspergillus fumigatus*, including the *Aspergillus viridinutans* species complex, are increasingly reported to be causes of invasive aspergillosis. Their identification is clinically relevant, as these species frequently have intrinsic resistance to common antifungals. We evaluated the susceptibilities of 90 environmental and clinical isolates from the *A. viridinutans* species complex, identified by DNA sequencing of the calmodulin gene, to seven antifungals (voriconazole, posaconazole, itraconazole, amphotericin B, anidulafungin, micafungin, and caspofungin) using the reference European Committee on Antimicrobial Susceptibility Testing (EUCAST) method. The majority of species demonstrated elevated MICs of voriconazole (geometric mean [GM] MIC, 4.46 mg/liter) and itraconazole (GM MIC, 9.85 mg/liter) and had variable susceptibility to amphotericin B (GM MIC, 2.5 mg/liter). Overall, the MICs of posaconazole and the minimum effective concentrations of echinocandins were low. The results obtained by the EUCAST method were compared with the results obtained with Sensititre YeastOne (YO) panels. Overall, there was 67% agreement (95% confidence interval [CI], 62 to 72%) between the results obtained by the EUCAST method and those obtained with YO panels when the results were read at 48 h and 82% agreement (95% CI, 78 to 86%) when the results were read at 72 h. There was a significant difference in agreement between antifungals; agreement was high for amphotericin B, voriconazole, and posaconazole (70 to 86% at 48 h and 88 to 93% at 72 h) but was very low for itraconazole (37% at 48 h and 57% at 72 h). The agreement was also variable between species, with the maximum agreement being observed for *A. felis* isolates (85 and 93% at 48 and 72 h, respectively). Elevated MICs of voriconazole and itraconazole were cross-correlated, but there was no correlation between the other azoles tested.

KEYWORDS cryptic species, *Aspergillus felis*, *Aspergillus udagawae*, amphotericin B, voriconazole, posaconazole, itraconazole, echinocandins

Invasive aspergillosis (IA) is one of the major mold infections, with IA resulting in high fatality rates among severely immunocompromised patients (1). *Aspergillus fumigatus* is the main etiologic agent of IA (2). Other *Aspergillus* species belonging to *Aspergillus* section *Fumigati* may occasionally cause IA, and most of them have been described in

Received 14 September 2017 Returned for modification 15 November 2017 Accepted 28 January 2018

Accepted manuscript posted online 5 February 2018

Citation Lyskova P, Hubka V, Svobodova L, Barrs V, Dhand NK, Yaguchi T, Matsuzawa T, Horie Y, Kolarik M, Dobias R, Hamal P. 2018. Antifungal susceptibility of the *Aspergillus viridinutans* complex: comparison of two *in vitro* methods. *Antimicrob Agents Chemother* 62:e01927-17. <https://doi.org/10.1128/AAC.01927-17>.

Copyright © 2018 American Society for Microbiology. All Rights Reserved.

Address correspondence to Pavlina Lyskova, pavlina.lyskova@zuusti.cz.

P.L., V.H., L.S., and V.B. contributed equally to this article.

the last 2 decades with the advent of molecular genetic methods (3). These species are frequently referred to as cryptic because they are morphologically similar to each other or to *A. fumigatus* and are often misidentified using conventional identification techniques (4–6). Misdiagnosis may have important consequences, as these *A. fumigatus*-related species often display some level of resistance to azoles and other antifungal drugs. *Aspergillus lentulus*, *A. udagawae*, *A. viridinutans*, and *A. thermomutatus* (*Neosartorya pseudofischeri*) have been most commonly associated with refractory cases of IA (3). Therefore, accurate species identification and antifungal susceptibility testing are required to guide therapy since the MICs of most antifungal drugs are higher for cryptic species than for *A. fumigatus sensu stricto* and azole cross-resistance can occur (3, 7, 8).

The *A. viridinutans* species complex contains morphologically similar, soil-inhabiting species that have been increasingly reported in recent years to be opportunistic human and animal pathogens with a clinical spectrum, including IA, chronic IA, and keratitis in humans, sino-orbital aspergillosis in cats, and disseminated IA in dogs (3, 5, 6, 9). This complex currently encompasses 10 species, and at least 6 of them are clinically relevant (5, 6, 9–13). Compared to the MICs for *A. fumigatus*, the MICs of a wider variety of antifungal agents for *A. viridinutans* species complex species are elevated (14). Despite the relatively high number of recently reported clinical cases, the antifungal susceptibility profiles of a large data set of reliably identified species have not been tested.

The aim of this study was to evaluate the *in vitro* antifungal susceptibility patterns of the *A. viridinutans* species complex to seven antifungals (voriconazole, itraconazole, posaconazole, amphotericin B, anidulafungin, micafungin, and caspofungin). Ninety isolates originating from human and animal specimens and from various environmental sources were tested. The results obtained by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) reference method were compared with those obtained with the widely used commercial Sensititre YeastOne (YO) panels to verify its applicability for antifungal susceptibility testing of cryptic species of *A. fumigatus*.

RESULTS

Molecular identification of isolates. The phylogenetic analyses performed using the maximum likelihood (ML) and Bayesian inference (BI) methods grouped the 90 isolates into several monophyletic and well-supported clades or single-isolate lineages (Fig. 1) corresponding to *A. udagawae* ($n = 17$), *A. wyomingensis* ($n = 15$), *A. arcoverdensis* ($n = 13$), *A. pseudoviridinutans* ($n = 7$), *A. aureolus* ($n = 3$), *A. siamensis* ($n = 2$), and *A. viridinutans* ($n = 1$). Five environmental isolates (IFM 57290, IFM 57291, CCF 4659, CCF 4660, CCF 4661) which did not produce ascospores and had green colonies were clustered as a sister branch to homothallic *A. aureolus* isolates with yellow colonies. Despite the close phylogenetic relationships, it is unlikely that these strains represent *A. aureolus* due to significant morphological differences and a heterothallic reproductive strategy. A new species status (*A. acensis* sp. nov.) is currently proposed for these strains (49). The ex-type strains of *A. felis*, *A. pseudofelis*, and *A. parafelis* clustered in one robust clade, and the phylogenetic analysis did not allow a clear distinction between these three species. Multiple gene phylogeny and *in vitro* mating experiment data indicate that these taxa are conspecific (49). Therefore, we considered all isolates belonging to this clade to be *A. felis*.

Antifungal susceptibility testing. The MIC ranges, MIC₅₀S, MIC₉₀S, and geometric mean (GM) MIC values obtained by the EUCAST reference method are shown in Table 1. The majority of the tested strains showed elevated MICs of itraconazole and voriconazole and frequently also elevated MICs of amphotericin B. The GM MIC of voriconazole ranged from 2.52 mg/liter for *A. aureolus* to 5.81 mg/liter for *A. arcoverdensis*. One-half of the strains of all species except *A. arcoverdensis* were inhibited by a voriconazole concentration of ≤ 4 mg/liter. High MICs were observed for itraconazole, with the GM MIC being 9.85 mg/liter and the MIC₅₀ being >8 mg/liter for all 90 isolates. In contrast, low MICs were determined for posaconazole, the GM MIC of which ranged

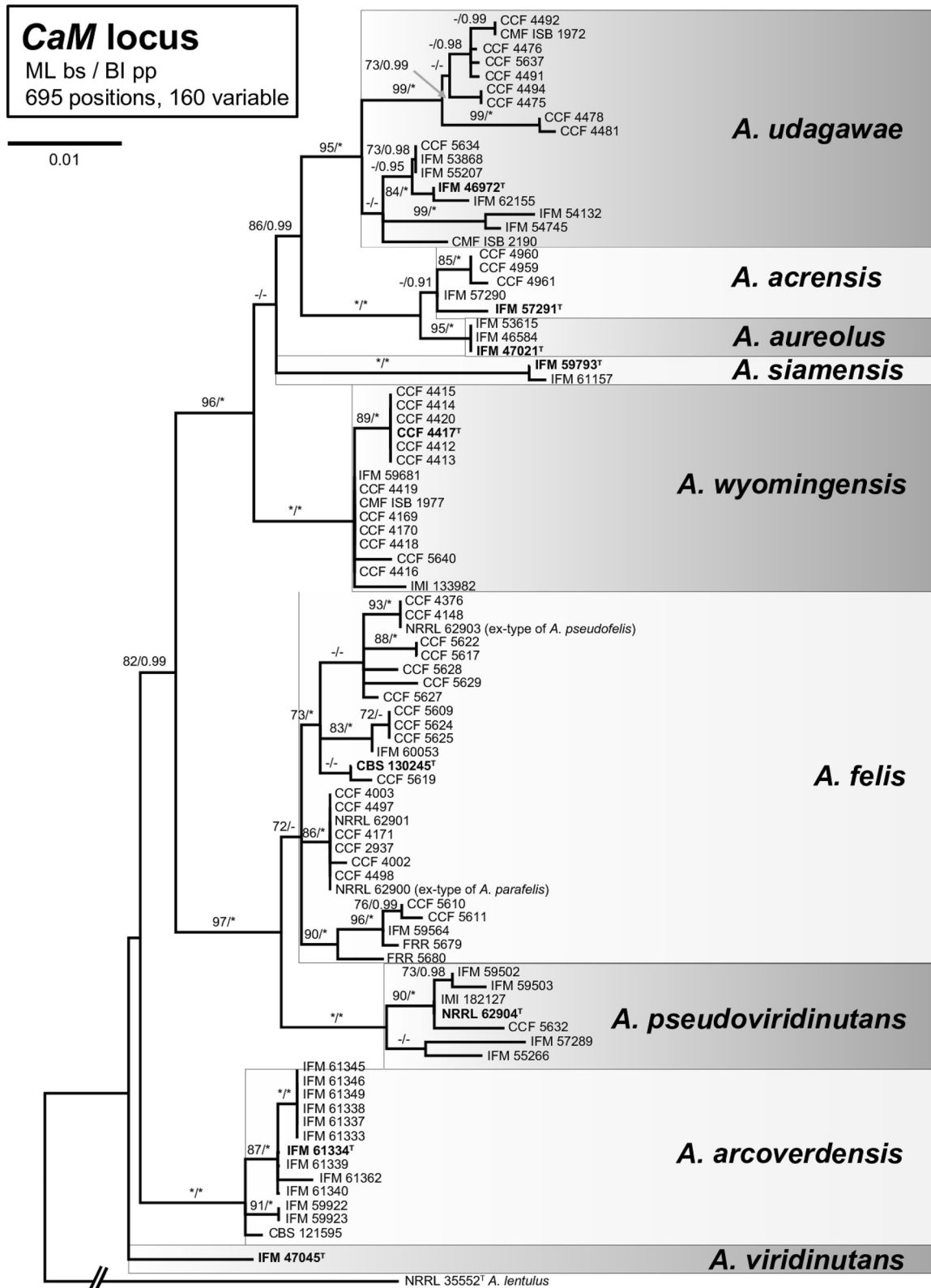


FIG 1 A 50% majority rule consensus maximum likelihood tree based on partial calmodulin gene (*CaM*) sequences shows the relationships of the 90 tested isolates belonging to the *Aspergillus viridinutans* complex. The maximum likelihood bootstrap proportion and Bayesian posterior probability are appended to the nodes; only bootstrap proportions of $\geq 70\%$ and posterior probabilities of $\geq 90\%$ are shown; lower levels of support are indicated with a hyphen, whereas asterisks indicate full support (a bootstrap proportion of 100% or a posterior probability of 1.00); ex-type strains are designated in boldface with a superscript T. The tree is rooted with *A. lentulus* NRRL 35552.

TABLE 1 Antifungal susceptibilities of 90 strains from the *Aspergillus viridinutans* complex by EUCAST reference method and percent agreement with Sensititre YeastOne panel^a

Species (no. of isolates)	Test agent	MIC by EUCAST (mg/liter)				% agreement with YeastOne panel			
		Range	GM	50%	90%	Range at 48 h	48 h	Range at 72 h	72 h
<i>A. felis</i> (27)	VRC	1 to >8	4.79	4	>8	0.5 to 4	100	1 to >8	100
	ITC	1 to >8	13.3	>8	>8	0.03 to >16	44.4	0.25 to >16	70.4
	POS	0.125 to 2	0.39	0.5	1	0.03 to 1	96.3	0.06 to 0.5	100
	AMB	0.25 to 8	1.63	2	8	0.25 to 4	100	1 to 8	100
	AFG	≤0.0312 to 0.125	0.03	≤0.0312	0.0625	ND	ND	ND	ND
	CAS	≤0.0312 to 8	0.08	≤0.0312	1	ND	ND	ND	ND
	MFG	≤0.0312 to 0.0625	0.03	≤0.0312	0.0625	ND	ND	ND	ND
Total agreement						85.2		92.6	
<i>A. udagawae</i> (17)	VRC	1 to >8	4	4	8	0.12 to 4	76.5	0.25 to 8	94.1
	ITC	0.25 to >8	7.37	>8	>8	0.03 to >16	35.3	0.03 to >16	52.9
	POS	0.0625 to 4	0.31	0.25	1	0.015 to 0.12	58.8	0.015 to 0.25	82.4
	AMB	1 to >8	3.54	8	8	0.5 to 4	76.5	0.5 to 8	88.2
	AFG	≤0.0312 to 0.25	0.04	≤0.0312	0.0625	ND	ND	ND	ND
	CAS	≤0.0312 to 2	0.07	≤0.0312	0.25	ND	ND	ND	ND
	MFG	≤0.0312 to 2	0.05	≤0.0312	0.0625	ND	ND	ND	ND
Total agreement						61.8		79.4	
<i>A. wyomingensis</i> (15)	VRC	4 to 8	4.2	4	4	0.12 to 2	33.3	0.5 to 2	86.7
	ITC	1 to >8	9.2	>8	>8	<0.015 to 0.25	0	0.03 to >16	0
	POS	0.125 to 0.25	0.16	0.125	0.25	0.008 to 0.5	46.7	0.015 to 1	80
	AMB	1 to >16	1.3	2	8	0.12 to 4	60	0.25 to 8	73.3
	AFG	≤0.0312 to 0.0625	0.03	≤0.0312	≤0.0312	ND	ND	ND	ND
	CAS	≤0.0312 to 0.25	0.04	≤0.0312	0.25	ND	ND	ND	ND
	MFG	≤0.0312 to 0.0625	0.03	≤0.0312	≤0.0312	ND	ND	ND	ND
Total agreement						35		60	
<i>A. arcoverdensis</i> (13)	VRC	4 to 8	5.81	8	8	0.25 to 2	53.8	1 to 4	84.6
	ITC	4 to >8	12.3	>8	>8	0.015 to >16	61.5	0.5 to >16	84.6
	POS	0.125 to 2	0.45	0.5	1	0.008 to 0.25	53.8	0.06 to 0.5	84.6
	AMB	0.5 to 8	1.6	2	2	0.25 to 2	84.6	1 to 2	100
	AFG	≤0.0312 to 0.125	0.03	≤0.0312	≤0.0312	ND	ND	ND	ND
	CAS	≤0.0312 to 0.25	0.05	≤0.0312	0.125	ND	ND	ND	ND
	MFG	≤0.0312 to 0.0625	0.03	≤0.0312	0.0625	ND	ND	ND	ND
Total agreement						63.5		88.5	
<i>A. pseudoviridinutans</i> (7)	VRC	4 to >8	5.3	4	>8	1 to 4	100	1 to 8	100
	ITC	>8	ND	>8	>8	>16	100	>16	100
	POS	0.125 to 1	0.4	0.5	1	0.12 to 0.25	100	0.25 to 1	100
	AMB	0.5 to 8	2	2	8	1 to 2	100	1 to 4	85.7
	AFG	≤0.0312 to 0.125	0.04	≤0.0312	0.125	ND	ND	ND	ND
	CAS	≤0.0312 to 0.25	0.05	≤0.0312	0.25	ND	ND	ND	ND
	MFG	≤0.0312 to 0.125	0.04	≤0.0312	0.125	ND	ND	ND	ND
Total agreement						100		96.4	
<i>A. acrensis</i> (5)	VRC	1 to 4	3.3	4	4	1 to 4	100	4 to 8	100
	ITC	1 to 4	3.3	4	4	0.06 to 0.12	0	0.12 to >16	40
	POS	0.125 to 1	0.38	0.5	1	0.03 to 0.06	40	0.06 to 0.12	80
	AMB	1 to 8	2	2	8	2 to 4	100	2 to 4	100
	AFG	≤0.0312 to 0.0625	0.04	≤0.0312	0.0625	ND	ND	ND	ND
	CAS	≤0.0312 to 0.25	0.125	0.125	0.25	ND	ND	ND	ND
	MFG	≤0.0312 to 0.0625	0.04	≤0.0312	0.0625	ND	ND	ND	ND
Total agreement						60		80	
<i>A. aureolus</i> (3)	VRC	1 to 4	2.52	ND	ND	0.25 to 1	ND	1 to 4	ND
	ITC	8 to >8	12.7	ND	ND	0.12 to 0.25	ND	>16	ND
	POS	0.125 to 0.5	0.25	ND	ND	0.06 to 0.12	ND	0.12 to 0.25	ND
	AMB	0.5 to 1	0.79	ND	ND	0.25 to 1	ND	1 to 2	ND
	AFG	≤0.0312 to 1	0.1	ND	ND	ND	ND	ND	ND
	CAS	≤0.0312 to 0.125	0.05	ND	ND	ND	ND	ND	ND
	MFG	≤0.0312 to 1	0.1	ND	ND	ND	ND	ND	ND
Total agreement						66.7		100	

(Continued on next page)

TABLE 1 (Continued)

Species (no. of isolates)	Test agent	MIC by EUCAST (mg/liter)				% agreement with YeastOne panel			
		Range	GM	50%	90%	Range at 48 h	48 h	Range at 72 h	72 h
<i>A. siamensis</i> (2)	VRC	4	4	ND	ND	0.5 to 1	ND	1 to 2	ND
	ITC	4	4	ND	ND	0.03 to 0.12	ND	0.06 to 0.25	ND
	POS	0.125 to 0.25	0.18	ND	ND	0.03 to 0.06	ND	0.03 to 0.12	ND
	AMB	0.25 to 1	0.5	ND	ND	1 to 2	ND	1 to 4	ND
	AFG	≤0.0312 to 0.25	0.09	ND	ND	ND	ND	ND	ND
	CAS	0.25 to 0.125	0.18	ND	ND	ND	ND	ND	ND
	MFG	ND	0.03	ND	ND	ND	ND	ND	ND
Total agreement								37.5	50
<i>A. viridinutans</i> (1)	VRC	4	ND	ND	ND	0.25	ND	0.5	ND
	ITC	4	ND	ND	ND	<0.015	ND	0.03	ND
	POS	0.125	ND	ND	ND	<0.008	ND	0.015	ND
	AMB	1	ND	ND	ND	0.5	ND	0.5	ND
	AFG	≤0.0312	ND	ND	ND	ND	ND	ND	ND
	CAS	0.0625	ND	ND	ND	ND	ND	ND	ND
	MFG	≤0.0312	ND	ND	ND	ND	ND	ND	ND
Total agreement								25	25
All isolates (90)	VRC	1 to >8	4.46	4	8	0.12 to 4	74.4	0.25 to >8	93.3
	ITC	0.25 to >8	9.85	>8	>8	0.015 to >16	36.7	0.03 to >16	56.7
	POS	0.0625 to 4	0.31	0.25	1	0.008 to 1	70	0.015 to 1	87.8
	AMB	0.25 to >16	2.5	2	8	0.12 to 4	85.6	0.25 to 8	91.1
	AFG	≤0.0312 to 1	0.04	≤0.0312	0.0625	ND	ND	ND	ND
	CAS	≤0.0312 to 8	0.05	≤0.0312	0.25	ND	ND	ND	ND
	MFG	≤0.0312 to 2	0.05	≤0.0312	0.0625	ND	ND	ND	ND
Overall agreement of methods (all antifungals)								66.7	82.2

^aThe GM and percent agreement were calculated only when there was a minimum of 5 isolates for a species. VRC, voriconazole; ITC, itraconazole; POS, posaconazole; AMB, amphotericin B; AFG, anidulafungin; CAS, caspofungin; MFG, micafungin; ND, not determined.

from 0.16 mg/liter for *A. wyomingensis* to 0.45 mg/liter for *A. arcoverdensis*. Susceptibility to amphotericin B was variable across the tested species; half of the isolates of each species were inhibited by a concentration of ≤2 mg/liter, and the highest MICs were obtained for *A. udagawae*. The minimum effective concentrations (MECs) determined for the echinocandins (anidulafungin, micafungin, and caspofungin) were low for almost all isolates, and the GM MIC was 0.04 mg/liter for anidulafungin, 0.05 mg/liter for caspofungin, and 0.05 mg/liter for micafungin.

The percent agreement of the results obtained by the EUCAST method and with the YO panels after 48 h (YO48) and 72 h (YO72) is shown in Table 1. Overall, there was 67% agreement (95% confidence interval [CI], 62% to 72%) between the results obtained by the EUCAST method and with the YO panels when they were read at 48 h and 82% agreement (95% CI, 78% to 86%) when they were read at 72 h. The agreement was significantly different for different antifungals (Table 2), with the maximum agreement being observed for voriconazole and amphotericin B (93% and 91%, respectively, at 72 h). For all antifungals, most of the MIC values obtained with YO48 were noticeably lower than those obtained by the reference EUCAST method, and consequently, the percent agreement for voriconazole, itraconazole, posaconazole, and amphotericin B when the results were read at 48 h was lower (74.4, 36.7, 70, and 85.6% agreement, respectively) than that when the results were read at 72 h (93.3, 56.7, 87.8, and 91.1% agreement, respectively). The worst correlation was detected for itraconazole in some species, and the lowest agreement with the reference method was detected for *A. wyomingensis*, i.e., 0% after 48 h and 72 h (Table 1). In contrast, agreement was 100% for all isolates of *A. pseudoviridinutans*, which had highly elevated MICs of this antifungal agent (MIC > 8 mg/liter), and for *A. arcoverdensis* (84.6%) after 72 h. Logistic regression results indicated that *A. felis* isolates had an eight times increased likelihood

TABLE 2 Unconditional logistic regression analyses conducted to investigate agreement between EUCAST reference method and Sensititre YeastOne panel for different antifungals and species^a

Variable	48 h				72 h			
	OR	95% CI	LSM	SE	OR	95% CI	LSM	SE
Antifungals								
Amphotericin B	2.03	0.97–4.43	0.86	0.04	0.73	0.23–2.20	0.91	0.03
Itraconazole	0.20	0.10–0.37	0.37	0.05	0.09	0.03–0.22	0.57	0.05
Posaconazole	0.80	0.41–1.54	0.70	0.05	0.51	0.17–1.42	0.88	0.03
Voriconazole ^b	1.00		0.74	0.05	1.00		0.93	0.03
Species ^c								
<i>A. felis</i>	10.68	5.15–23.22	0.85	0.03	8.33	3.57–21.38	0.93	0.03
<i>A. acrensis</i>	2.79	1.00–8.16	0.60	0.11	2.67	0.86–10.18	0.80	0.09
<i>A. arcovirdensis</i>	3.23	1.50–7.12	0.63	0.07	5.11	1.99–15.00	0.88	0.04
<i>A. siamensis</i>	1.11	0.21–5.00	0.38	0.17	0.67	0.15–3.06	0.50	0.18
<i>A. udagawae</i>	3.00	1.47–6.26	0.62	0.06	2.57	1.19–5.73	0.79	0.05
<i>A. wyomingensis</i> ^b	1.00		0.35	0.06	1.00		0.60	0.06

^aFor the definition of agreement, see Materials and Methods. OR, odds ratio; CI, confidence interval; LSM, least-squares mean; SE, standard error.

^bReference categories for odds ratios.

^cThe results for three species, *A. pseudoviridinutans*, *A. viridinutans*, and *A. aureolus*, were excluded from this analysis because of zero or low frequencies in cells of the contingency table of species with the agreement outcome.

(odds ratio [OR], 8) of agreement between the results obtained by the EUCAST method and with the YO panels than *A. wyomingensis* isolates. The likelihood of agreement between the EUCAST method and the YO panels was the lowest for *A. siamensis* isolates and the highest for *A. felis* isolates (Table 2).

The azole MIC data cross-tabulated in Table 3 indicate a correlation between the elevated MIC values of voriconazole and itraconazole, since 81 of 90 isolates had elevated MIC values of both drugs and the McNemar test result was significant ($P < 0.001$). In contrast, no correlation between the MIC values of the other azoles was observed (Table 3); 81 of 87 isolates with low MICs of posaconazole had elevated MICs of itraconazole, and 83 of 87 isolates with low MICs of posaconazole had elevated MICs of voriconazole. This lack of a correlation was confirmed by significant McNemar test results ($P < 0.001$) and very low kappa values (Table 3).

DISCUSSION

Our data show that species from the *A. viridinutans* species complex have variable MICs of amphotericin B and elevated MICs of itraconazole and voriconazole (Table 1). The most elevated MICs were observed for itraconazole, while almost all *A. viridinutans* species complex isolates had low MICs and MECs of posaconazole and echinocandins.

TABLE 3 Evaluation of cross-correlation of elevated MICs of azoles^a

Species	Antifungal agent	Kappa value (SE)		
		VRC	ITC	POS
All species	VRC	1	0.15 (0.17)	0.0032 (0.002)
	ITC		1	0.0049 (0.003)
	POS			1
<i>A. felis</i>	VRC	1	−0.039 (0.027)	0.003 (0.004)
	ITC		1	0.003 (0.004)
	POS			1
<i>A. udagawae</i>	VRC	1	−0.097 (0.077)	0.0078 (0.01)
	ITC		1	0.026 (0.03)
	POS			1

^aAn elevated MIC value was defined as an MIC of ≥ 2 mg/liter. VRC, voriconazole; ITC, itraconazole; POS, posaconazole.

Published clinical cases caused by *A. viridinutans* species complex isolates, the antifungal MICs (or MECs), and the clinical outcome of therapy are reviewed in Table 4. It was demonstrated that many representatives of the complex have elevated MICs of azoles (itraconazole and voriconazole) and amphotericin B (summarized in Table 4) (4, 5, 7–9, 14–31). Howard (32) reviewed data for some cryptic species and designated *A. viridinutans* and *A. felis* to be susceptible to amphotericin B, having reduced susceptibility to voriconazole and variable susceptibility to itraconazole, posaconazole, and echinocandins. The detection of elevated MICs of polyenes (amphotericin B), azoles (voriconazole, posaconazole, itraconazole), or echinocandins in *A. fumigatus*-like isolates during antifungal susceptibility testing may indicate the presence of cryptic species, as the majority of *A. fumigatus* isolates are susceptible to these antifungals (33, 34), although resistance to azoles has been increasingly reported in some countries (35). Most other investigators have reported low MECs of echinocandins, in accordance with our data (9, 14, 16, 18, 20, 21, 23, 26, 27), with the exception of Yaguchi et al. (7), who reported high MICs of micafungin obtained by the CLSI method (>16 mg/liter). Barrs et al. (9) examined the MICs of 13 *A. felis* isolates using YO48 and found MICs of itraconazole of less than 1 mg/liter (9). However, as shown in this study, a reading time after only 48 h is associated with a high degree of falsely low MICs (Table 1). Some other authors reported high MICs of itraconazole in isolates from the *A. viridinutans* complex, in agreement with the findings of our study (Table 4) (5, 16–18, 20, 24). Interestingly, we observed a paradoxical effect for itraconazole with YO48 and YO72 for many of the strains tested. According to the manufacturer's instructions (Trek Diagnostic Systems), this phenomenon should be ignored and the lower MIC should be recorded. However, according to our results, it seems that the phenomenon should not be ignored, because it may indicate elevated MICs of this antifungal.

The *A. viridinutans* species complex belongs to the section *Fumigati*, and morphological differentiation of these species from *A. fumigatus*, the most common agent of IA, requires expertise (2, 5, 8, 36). So-called cryptic species may remain unrecognized, especially in laboratories that still predominantly use only phenotypic identification. The frequency of cryptic section *Fumigati* species in clinical settings has been reported to be between 4 and 5% in some studies that used molecular methods for identification (2, 8). The recognition of cryptic species is important because some of them exhibit susceptibility profiles different from those of their well-known relatives. Cases of human and animal infections due to the *A. viridinutans* species complex reported previously were mostly attributed to *A. udagawae*, *A. felis*, and *A. viridinutans* (Table 4). The isolates responsible for many of these reported infections showed clinical resistance to antifungal therapy (Table 4), even though *in vitro* susceptibility testing showed low MICs of these antifungals (17, 18, 20). Some authors published descriptions of a positive clinical effect of therapy with posaconazole, posaconazole plus caspofungin, or amphotericin B plus caspofungin (17, 18, 20, 22). A case of human IA due to *A. udagawae* was successfully treated using voriconazole, and a feline infection was successfully treated using high doses of itraconazole (23, 25), while in other cases there was little or no effect when voriconazole or itraconazole was used for therapy (14, 17, 18, 20, 24, 25). Coelho et al. (20) reported clinical improvement when using posaconazole at the beginning of therapy, but later the therapy failed and the patient died. This could be due to either an advanced stage of infection or the development of resistance. The latter phenomenon is documented in *A. fumigatus* infections (37). Voriconazole is recommended for the first-line treatment of IA in humans (38), and when resistance to azoles is detected, therapy is switched to liposomal amphotericin B. If the rate of environmental resistance to *A. fumigatus* is high ($\geq 10\%$) in the region, combinations of voriconazole with echinocandin or liposomal amphotericin B are favored as initial therapy (38). Due to the apparent low frequency of cryptic *Aspergillus* species recognized in clinical practice, there are no solid clinical data to guide therapy (39). However, as antifungal susceptibility varies largely among fungal isolates and species, *in vitro* susceptibility testing for every isolate of a cryptic species is essential.

TABLE 4 Antifungal susceptibility, treatment response, and outcome for clinical isolates of *A. viridinutans* complex previously reported^a

Species (no. of isolates)	MIC or MEC (mg/liter)							Method	Antifungal agent (response to treatment) ^b	Outcome	Reference(s)
	AMB	ITC	VRC	POS	AFG	CAS	MFG				
<i>A. felis</i> (1) ^c	1	2					>16	CLSI			7
<i>A. felis</i> (2) ^d	0.5 to 1	16	4	0.25 to 0.5		1	0.03 to 0.2	CLSI			16
<i>A. felis</i> (1) ^e	1	>16	4	0.25	≤0.016			CLSI	ITC (1), VRC (1), POS + CAS (1), POS (3, 1)	Died	9
<i>A. felis</i> (13)	0.25 to 1	0.03 to 1	0.25 to 4	0.03 to 1	0.015	0.008 to 2	0.008 to 0.015	YO48			20
<i>A. felis</i> (1) ^f	4	4	4	0.5				CLSI	MFG (1), VRC (1), AMB + CAS (1)	Poor	24
<i>A. felis</i> (1) ^g	0.064	2	0.5	0.064				Etest	No treatment (1)	Died (cat)	25
<i>A. felis</i> (1)	1	1	4	0.5	<0.03	1	<0.03	EUCAST	AMB + CAS (1)	Died	26, 28
<i>A. felis</i> (5) ^h	1 to 2	8 to >16	8	0.5				CLSI		Died	12
<i>A. felis</i> (1)/ <i>A. pseudoviridinutans</i> (3) ⁱ	0.5 ^j	2/	4/	1 to 2				CLSI	VRC (1), AMB (1)	Died	29
<i>A. felis</i> (1)	2	0.125	0.5			0.25		EUCAST	VRC (1), POS + CAS (3)	Survived	30
<i>A. pseudoviridinutans</i> (2) ^k	4 to 8	1	1	0.06		0.06 to 0.25		CLSI	VRC (1), AMB + CAS (1), POS (1), POS + CAS + 5FC (1)	Died	17
<i>A. pseudoviridinutans</i> (1) ^l	1	2	4	≤0.016 to 0.5				CLSI	FLC + VRC (1), VRC + AMB + NAT + TER (1)	Survived (surgery)	14
<i>A. udagawae</i> (1)	>32	0.5	0.25	0.25			0.015	Etest	ITC + AMB (1), AMB + CAS (1)	Died (cat)	19
<i>A. udagawae</i> (3)	1 to 2	0.25 to 2	0.5 to 2	0.125 to 0.25				CLSI		Died (cat)	8
<i>A. udagawae</i> (4)	1	1	2	0.25		1	≤0.015	CLSI	VRC (1), VRC + CAS (1), POS (1), AMB + VRC (2), AMB (2), AMB + CAS (3)	Survived	18
	2	1	2	0.25		0.25	≤0.015		ITC (1), POS (3)	Survived	18
	2	4	>16	0.5		0.5	≤0.015		POS (1), VRC + CAS (1), VRC + CAS + AMB + TER + 5FC (1)	Dissemination to brain, died	18
	0.5 to 1	1	1 to 4	0.5		0.25 to 0.5	≤0.015		ITC(1), AMB (1), VRC + CAS (1), POS + MFG + AMB + ITC (1), CAS (2), VRC + CAS (2)	Dissemination, died	18
<i>A. udagawae</i> (1)	0.125	0.25	8	0.25	0.016	0.5	0.25	CLSI	AMB (1), AMB + ITC (1), ITR (2)	Survived (surgery)	21
<i>A. udagawae</i> (1)	2	0.5	0.5	0.12 to 0.25		0.03 to 0.06	0.03 to 0.12	CLSI	VRC (3)	Survived	23
<i>A. udagawae</i> (2)		1	1	0.5			≤0.016	CLSI	VRC + CAS (1)	Poor	24
<i>A. udagawae</i> (1)	>32	0.25	1					Etest	AMB + MFG + VRC (1)	Poor	24
<i>A. udagawae</i> (5)	0.05 to 4	0.25 to 1	2 to 4			0.03 to 0.06	0.03 to 0.12	CLSI	ITC (3)	Survived (cat)	25
<i>A. udagawae</i> (9)	1 ^l	0.5 ^l	8 ^l					CLSI			27
<i>A. udagawae</i> (1)	4	0.5	2					CLSI	VOR (1)	Died	29
<i>A. viridinutans</i> (1) ^m	1	0.5	1				>16	CLSI			31
<i>A. viridinutans</i> (1) ^m	0.25	1	1					CLSI			7

^aMEC, minimal effective concentration; AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; POS, posaconazole; AFG, anidulafungin; CAS, caspofungin; MFG, micafungin; POS, posaconazole; TER, terbinafine; NAT, natamycin; YO48, Sensititre YeastOne panel after 48 h.

^b1, none/progressive disease; 2, weak response; 3, clinical improvement.

^cIsolate IFM 54303, originally listed as *A. viridinutans*, was reidentified in this study as *A. felis*.

^dStrains CM-3147 (= NRRL 62900 = CCF 4895) and CM-4518 (= NRRL 62902), listed as *A. viridinutans* in the original publication, are *A. felis* by Barrs et al. (9) and was included in the present study.

^eThis strain (NRRL 62901 = CM-5623 = CCF 4896 = CCF 4557) was later reidentified as *A. felis* by Barrs et al. (9) and was included in the present study.

^fThis strain is *A. felis* on the basis of current taxonomy.

^gThe strain, listed as *A. viridinutans*, is *A. felis* on the basis of current taxonomy.

^hIsolates of *A. pseudofelis* and *A. parafelis* were considered conspecific with *A. felis* in this study.

ⁱThe *A. viridinutans* strains included in the study of Tamiya et al. (29) are *A. felis* (IFM 54303) and three strains of *A. pseudoviridinutans* (IFM 55266, IFM 59502, and IFM 59503); all strains were tested in the present study.

^jThe value represents the MIC₅₀.

^kIsolates NIHAV1 and NIHAV2, listed as *A. viridinutans*, were subsequently described as *A. pseudoviridinutans* by Sugui et al. (12).

^lThis strain (IFM 59502), listed as *A. viridinutans*, was reidentified as *A. pseudoviridinutans* in the present study.

^mEx-type strain of *A. viridinutans*.

Conclusions. In this study, most *A. viridinutans* species complex isolates demonstrated elevated MICs of itraconazole and voriconazole *in vitro*, and susceptibility to amphotericin B was variable. In contrast, posaconazole and echinocandins had potent *in vitro* activity against *A. viridinutans* species complex isolates. There were no clear antifungal susceptibility patterns between species, and intraspecific variation was usually high. This fact highlights the need to use reliable methods for MIC determinations over correct identification to a species level. However, the identification to a level of species complex and differentiation from *A. fumigatus* are also important due to different susceptibility patterns. The agreement between the Sensititre YeastOne commercial method and the EUCAST method was high, especially for the most clinically important species in the *A. viridinutans* complex, i.e., *A. felis*, *A. udagawae*, and *A. pseudoviridinutans*, in contrast to infrequently pathogenic or nonpathogenic species, such as *A. wyomingensis* and *A. siamensis*. Better agreement between the methods was usually achieved with a reading time of 72 h than with one of 48 h. Importantly, however, the YeastOne panel frequently did not detect elevated MICs of itraconazole.

MATERIALS AND METHODS

Antifungal agents. Anidulafungin and voriconazole in powder form were procured from Pfizer Pharmaceutical Group (New York, NY, USA), micafungin from Astellas Pharma Inc. (Tokyo, Japan), caspofungin and posaconazole from Merck Sharp & Dohme Research Laboratories (Rahway, NJ, USA), and itraconazole and amphotericin B from Sigma-Aldrich (Prague, Czech Republic).

Organisms. A total of 90 *Aspergillus* isolates from the *A. viridinutans* species complex were collected from various environmental and clinical sources worldwide. All the ex-type strains of currently described species in the *A. viridinutans* complex were also examined. Information on the isolation source of all isolates is provided in Table S1 in the supplemental material.

Molecular methods. An ArchivePure DNA yeast and Gram2+ kit (5 Prime Inc., Gaithersburg, MD, USA) was used for DNA isolation from 7-day-old cultures according to the manufacturer's instructions, as updated by Hubka et al. (40). The calmodulin gene (*CaM*) was amplified using forward primer CF1M or CF1L and reverse primer CF4 (41). The PCR conditions were those described by Hubka et al. (42). PCR product purification followed the protocol of R blova et al. (43). Automated sequencing was performed at the Macrogen Sequencing Service (Amsterdam, The Netherlands) using both terminal primers.

Sequences were inspected and assembled using the BioEdit (v.7.2.5) program (www.mbio.ncsu.edu/BioEdit/bioedit.html). Alignment was performed using the G-INS-i option implemented in the MAFFT (v.7) program (44). The alignment was trimmed and then analyzed using maximum likelihood (ML) and Bayesian inference (BI) analyses. The analyses involved 91 nucleotide sequences and 695 positions, of which 160 were variable and 104 were parsimony informative. Suitable partitioning scheme and substitution models (according to the Bayesian information criterion) for analyses were selected using the greedy strategy implemented in the PartitionFinder (v.1.1.1) program (45) with settings allowing introns, exons, and codon positions to be independent data sets. The optimal partitioning scheme for ML analysis divided the data set into four partitions with the following substitution models: K80+G substitution models were proposed for *CaM* introns, the F81 model was proposed for the 1st codon positions, the F81 model was proposed for the 2nd codon positions, and the HKY model was proposed for the 3rd codon positions. The ML tree was constructed with the IQ-TREE (v.1.4.4) program (46), with nodal support being determined by nonparametric bootstrapping with 1,000 replicates. *Aspergillus lentulus* NRRL 35552 was used as the outgroup. Bayesian posterior probabilities were calculated using the MrBayes (v.3.2.6) program (47). Optimal partitioning scheme and substitution models were selected as described above. The optimal partitioning scheme for BI analysis divided the data set into four partitions with the following substitution models: K80+G substitution models were proposed for *CaM* introns, the F81 model was proposed for the 1st codon positions, the F81 model was proposed for the 2nd codon positions, and the HKY model was proposed for the 3rd codon positions. The analyses ran for 10⁷ generations, two parallel runs with four chains each were used, every 1,000th tree was retained, and the first 25% of the trees was discarded as burn-in.

Susceptibility testing. The broth microdilution method was performed according to EUCAST document E.Def 9.3 (48). The isolates were incubated at 35°C on potato dextrose agar (Trios, Prague, Czech Republic). Inoculum suspensions were prepared from 7- to 14-day-old colonies (to achieve acceptable sporulation), the suspensions were filtered using sterile nylon filters with an 11-μm pore size (Merck, Prague, Czech Republic), and a spectrophotometer (model Spekol 11; Carl Zeiss, Jena, Germany) was used to adjust the suspension to a concentration of 2 × 10⁶ to 5 × 10⁶ conidia/ml (optical density, 0.09 to 0.11), equivalent to a McFarland 0.5 standard. Microplates were incubated at 35°C in ambient air for 48 h. MICs were determined for amphotericin B and the azoles, and minimum effective concentrations (MECs) were determined for the echinocandins.

Assays with Sensititre YeastOne (YO) panels (Trek Diagnostic System Ltd., East Grinstead, UK) were performed according to the manufacturer's instructions. The MICs for *Aspergillus* species were determined to be the lowest concentration with a blue color. The MICs of amphotericin B, voriconazole, itraconazole, and posaconazole were evaluated with YO panels after 48 h (YO48) and after 72 h (YO72)

of incubation. Echinocandins were not evaluated with the YO panels because MEC endpoints should be determined after 24 h according to the manufacturer's instructions. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality control strains.

Data analysis. For the EUCAST method, MIC ranges and the corresponding GM MIC values were determined for each species, antifungal drug, and incubation time. The MIC₅₀s and MIC₉₀s were determined for species represented by at least five isolates in our data set. Discrepancies among MIC endpoints of no more than 2-fold dilutions were used to calculate the percent agreement that was determined for each combination of isolate, drug, and incubation time for species represented by at least five isolates in our data set.

Statistical analysis. Descriptive statistics were calculated using Microsoft Excel software. Further statistical analyses were conducted using SAS (v.9.4) software (SAS Institute Inc., Cary, NC, USA). For GM MIC calculation, MIC values of <0.015 mg/liter were set at 0.008 mg/liter and MIC values of >16 mg/liter were set at 32 mg/liter, while MEC values of <0.008 mg/liter were set at 0.004 mg/liter and MEC values of >8 mg/liter were set at 16 mg/liter. For calculation of agreement between the results obtained with the YO panel and by the EUCAST method, extreme values were treated similarly. Logistic regression analyses were conducted to evaluate differences in agreement between species and antifungals. Odds ratios (ORs), their 95% confidence intervals, least-squares means, and standard errors were determined. The correlation of elevated MIC values between azoles was tested by analyzing the MIC values for each pair of antifungal drugs. For this purpose, since no interpretive breakpoints are established, elevated MIC values were defined as MICs of ≥2 mg/liter. A cross-correlation between the MICs of different azoles was estimated by cross-tabulating the data, conducting McNemar tests, and calculating kappa statistics. All *P* values reported here are two-sided. Differences were considered statistically significant at a *P* value of ≤0.05.

Accession number(s). The sequences generated in this study were deposited into the EMBL (European Molecular Biology Laboratory) database under the accession numbers listed in Table S1.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.01927-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

This research was supported by the projects of the Charles University Grant Agency (GAUK 1130214), Charles University Research Centre program no. 204069, and project BIOCEV (CZ.1.05/1.1.00/02.0109), provided by the Ministry of Education, Youth and Sports of the Czech Republic and ERDF. Additional support was obtained from research grant no. IGA_LF_2017_031 and RVO: 61989592 (Palacky University, Olomouc, Olomouc, Czech Republic), and by a Thompson Fellowship from the University of Sydney.

We thank Milada Chudíčková and Alena Gabrielová for their invaluable assistance in the laboratory and Alena Nováková, Alena Kubátová, Stephen W. Peterson, Mark Wilson, Adrian Zelazny, and Maria Dolores Pinheiro for providing some cultures. We also thank Pfizer Pharmaceutical Group (New York, NY, USA) for providing voriconazole and anidulafungin, Astellas Pharma Inc. (Tokyo, Japan) for providing micafungin, and Merck Sharp & Dohme Research Laboratories (Rahway, NJ, USA) for providing caspofungin and posaconazole.

We declare that we have no conflict of interest.

REFERENCES

- Lin SJ, Schranz J, Teutsch SM. 2001. Aspergillois case-fatality rate: systematic review of the literature. *Clin Infect Dis* 32:358–366. <https://doi.org/10.1086/318483>.
- Alastruey-Izquierdo A, Mellado E, Peláez T, Pemán J, Zapico S, Alvarez M, Rodríguez-Tudela JL, Cuenca-Estrella M, FILPOP Study Group. 2013. Population-based survey of filamentous fungi and antifungal resistance in Spain (FILPOP study). *Antimicrob Agents Chemother* 57:3380–3387. <https://doi.org/10.1128/AAC.00383-13>.
- Lamoth F. 2016. *Aspergillus fumigatus*-related species in clinical practice. *Front Microbiol* 7:683. <https://doi.org/10.3389/fmicb.2016.00683>.
- Balajee SA, Gribskov JL, Hanley E, Nickle D, Marr KA. 2005. *Aspergillus lentulus* sp. nov., a new sibling species of *A. fumigatus*. *Eukaryot Cell* 4:625–632. <https://doi.org/10.1128/EC.4.3.625-632.2005>.
- Sugui JA, Vinh DC, Nardone G, Shea YR, Chang YC, Zelazny AM, Marr KA, Holland SM, Kwon-Chung KJ. 2010. *Neosartorya udagawae* (*Aspergillus udagawae*), an emerging agent of aspergillois: how different is it from *Aspergillus fumigatus*? *J Clin Microbiol* 48:220–228. <https://doi.org/10.1128/JCM.01556-09>.
- Nováková A, Hubka V, Dudová Z, Matsuzawa T, Kubátová A, Yaguchi T, Kolařík M. 2014. New species in *Aspergillus* section *Fumigati* from reclamation sites in Wyoming (USA) and revision of *A. viridinutans* complex. *Fungal Divers* 64:253–274. <https://doi.org/10.1007/s13225-013-0262-5>.
- Yaguchi T, Horie Y, Tanaka R, Matsuzawa T, Ito J, Nishimura K. 2007. Molecular phylogenetics of multiple genes on *Aspergillus* section *Fumigati* isolated from clinical specimens in Japan. *Jpn J Med Mycol* 48:37–46. <https://doi.org/10.3314/jjmm.48.37>.
- Balajee SA, Kano R, Baddley JW, Moser SA, Marr KA, Alexander BD, Andes D, Kontoyiannis DP, Perrone G, Peterson S, Brandt ME, Pappas PG, Chiller T. 2009. Molecular identification of *Aspergillus* species collected for the transplant-associated infection surveillance network. *J Clin Microbiol* 47:3138–3141. <https://doi.org/10.1128/JCM.01070-09>.
- Barrs VR, van Doorn TM, Houbraken J, Kidd SE, Martin P, Pinheiro MD,

- Richardson M, Varga J, Samson RA. 2013. *Aspergillus felis* sp. nov., an emerging agent of invasive aspergillosis in humans, cats, and dogs. PLoS One 8:e64871. <https://doi.org/10.1371/journal.pone.0064871>.
10. Eamvijarn A, Manoch L, Chamsawang C, Piasai O, Visarathanonth N, Luangsaard JJ, Kijjoo A. 2013. *Aspergillus siamensis* sp. nov. from soil in Thailand. Mycoscience 54:401–405. <https://doi.org/10.1016/j.myc.2013.01.005>.
 11. Barrs V, Beatty J, Dhand NK, Talbot J, Bell E, Abraham L, Chapman P, Bennett S, van Doorn T, Makara M. 2014. Computed tomographic features of feline sino-nasal and sino-orbital aspergillosis. Vet J 201: 215–222. <https://doi.org/10.1016/j.tvjl.2014.02.020>.
 12. Sugui JA, Peterson SW, Figat A, Hansen B, Samson RA, Mellado E, Cuenca-Estrella M, Kwon-Chung KJ. 2014. Genetic relatedness versus biological compatibility between *Aspergillus fumigatus* and related species. J Clin Microbiol 52:3707–3721. <https://doi.org/10.1128/JCM.01704-14>.
 13. Matsuzawa T, Takaki GMC, Yaguchi T, Okada K, Abliz P, Gonoi T, Horie Y. 2015. *Aspergillus arcovendensis*, a new species of *Aspergillus* section *Fumigati* isolated from Caatinga soil in state of Pernambuco, Brazil. Mycoscience 56:123–131. <https://doi.org/10.1016/j.myc.2014.04.006>.
 14. Shigeyasu C, Yamada M, Nakamura N, Mizuno Y, Sato T, Yaguchi T. 2012. Keratomycosis caused by *Aspergillus viridinutans*: an *Aspergillus fumigatus*-resembling mold presenting distinct clinical and antifungal susceptibility patterns. Med Mycol 50:525–528. <https://doi.org/10.3109/13693786.2012.658875>.
 15. Katz ME, Dougall AM, Weeks K, Cheetham BF. 2005. Multiple genetically distinct groups revealed among clinical isolates identified as atypical *Aspergillus fumigatus*. J Clin Microbiol 43:551–555. <https://doi.org/10.1128/JCM.43.2.551-555.2005>.
 16. Alcazar-Fuoli L, Mellado E, Alastruey-Izquierdo A, Cuenca-Estrella M, Rodriguez-Tudela JL. 2008. *Aspergillus* section *Fumigati*: antifungal susceptibility patterns and sequence-based identification. Antimicrob Agents Chemother 52:1244–1251. <https://doi.org/10.1128/AAC.00942-07>.
 17. Vinh DC, Shea YR, Jones PA, Freeman AF, Zelazny A, Holland SM. 2009. Chronic invasive aspergillosis caused by *Aspergillus viridinutans*. Emerg Infect Dis 15:1292–1294. <https://doi.org/10.3201/eid1508.090251>.
 18. Vinh DC, Shea YR, Sugui JA, Parrilla-Castellar ER, Freeman AF, Campbell JW, Pittaluga S, Jones PA, Zelazny A, Kleiner D, Kwon-Chung KJ, Holland SM. 2009. Invasive aspergillosis due to *Neosartorya udagawae*. Clin Infect Dis 49:102–111. <https://doi.org/10.1086/599345>.
 19. Kano R, Itamoto K, Okuda M, Inokuma H, Hasegawa A, Balajee SA. 2008. Isolation of *Aspergillus udagawae* from a fatal case of feline orbital aspergillosis. Mycoses 51:360–361. <https://doi.org/10.1111/j.1439-0507.2008.01493.x>.
 20. Coelho D, Silva S, Vale-Silva L, Gomes H, Pinto E, Sarmiento A, Pinheiro MD. 2011. *Aspergillus viridinutans*: an agent of adult chronic invasive aspergillosis. Med Mycol 49:755–759. <https://doi.org/10.3109/13693786.2011.556672>.
 21. Posteraro B, Mattei R, Trivella F, Maffei A, Torre A, De Carolis E, Posteraro P, Fadda G, Sanguinetti M. 2011. Uncommon *Neosartorya udagawae* fungus as a causative agent of severe corneal infection. J Clin Microbiol 49:2357–2360. <https://doi.org/10.1128/JCM.00134-11>.
 22. Barrs VR, Halliday C, Martin P, Wilson B, Krockenberger M, Gunew M, Bennett S, Koehlmeier E, Thompson A, Flegner R, Hocking A, Sleiman S, O'Brien C, Beatty JA. 2012. Sinonasal and sino-orbital aspergillosis in 23 cats: aetiology, clinicopathological features and treatment outcomes. Vet J 191:58–64. <https://doi.org/10.1016/j.tvjl.2011.02.009>.
 23. Gyotoku H, Izumikawa K, Ikeda H, Takazono T, Morinaga Y, Nakamura S, Imamura Y, Nishino T, Miyazaki T, Kakeya H, Yamamoto Y, Yanagihara K, Yasuoka A, Yaguchi T, Ohno H, Miyazaki Y, Kamei K, Kanda T, Kohno S. 2012. A case of bronchial aspergillosis caused by *Aspergillus udagawae* and its mycological features. Med Mycol 50:631–636. <https://doi.org/10.3109/13693786.2011.639036>.
 24. Escibano P, Peláez T, Muñoz P, Bouza E, Guinea J. 2013. Is azole resistance in *Aspergillus fumigatus* a problem in Spain? Antimicrob Agents Chemother 57:2815–2820. <https://doi.org/10.1128/AAC.02487-12>.
 25. Kano R, Shibahashi A, Fujino Y, Sakai H, Mori T, Tsujimoto H, Yanai T, Hasegawa A. 2013. Two cases of feline orbital aspergillosis due to *Aspergillus udagawae* and *A. viridinutans*. J Vet Med Sci 75:7–10. <https://doi.org/10.1292/jvms.12-0119>.
 26. Peláez T, Alvarez-Perez S, Mellado E, Serrano D, Valerio M, Blanco JL, Garcia ME, Munoz P, Cuenca-Estrella M, Bouza E. 2013. Invasive aspergillosis caused by cryptic *Aspergillus* species: a report of two consecutive episodes in a patient with leukaemia. J Med Microbiol 62:474–478. <https://doi.org/10.1099/jmm.0.044867-0>.
 27. Alastruey-Izquierdo A, Alcazar-Fuoli L, Cuenca-Estrella M. 2014. Antifungal susceptibility profile of cryptic species of *Aspergillus*. Mycopathologia 178:427–433. <https://doi.org/10.1007/s11046-014-9775-z>.
 28. Álvarez-Pérez S, Mellado E, Serrano D, Blanco JL, Garcia ME, Kwon M, Munoz P, Cuenca-Estrella M, Bouza E, Peláez T. 2014. Polyphasic characterization of fungal isolates from a published case of invasive aspergillosis reveals misidentification of *Aspergillus felis* as *Aspergillus viridinutans*. J Med Microbiol 63:617–619. <https://doi.org/10.1099/jmm.0.068502-0>.
 29. Tamiya H, Ochiai E, Kikuchi K, Yahiro M, Toyotome T, Watanabe A, Yaguchi T, Kamei K. 2015. Secondary metabolite profiles and antifungal drug susceptibility of *Aspergillus fumigatus* and closely related species, *Aspergillus lentulus*, *Aspergillus udagawae*, and *Aspergillus viridinutans*. J Infect Chemother 21:385–391. <https://doi.org/10.1016/j.jiac.2015.01.005>.
 30. Chong GM, Vonk AG, Meis JF, Dingemans GJH, Houbraken J, Hagen F, Gaajetaan GR, van Tegelen DWE, Simons GFM, Rijnders BJA. 2017. Interspecies discrimination of *A. fumigatus* and siblings *A. lentulus* and *A. felis* of the *Aspergillus* section *Fumigati* using the AsperGenius® assay. Diagn Microbiol Infect Dis 87:247–252. <https://doi.org/10.1016/j.diagmicrobio.2016.11.020>.
 31. Seki A, Yoshida A, Matsuda Y, Kawata M, Nishimura T, Tanaka J, Misawa Y, Nakano Y, Asami R, Chida K, Kikuchi K, Arai T. 2017. Fatal fungal endocarditis by *Aspergillus udagawae*: an emerging cause of invasive aspergillosis. Cardiovasc Pathol 28:14–17. <https://doi.org/10.1016/j.carpath.2017.02.002>.
 32. Howard SJ. 2014. Multi-resistant aspergillosis due to cryptic species. Mycopathologia 178:435–439. <https://doi.org/10.1007/s11046-014-9774-0>.
 33. Diekema DJ, Messer SA, Hollis RJ, Jones RN, Pfaller MA. 2003. Activities of caspofungin, itraconazole, posaconazole, ravuconazole, voriconazole, and amphotericin B against 448 recent clinical isolates of filamentous fungi. J Clin Microbiol 41:3623–3626. <https://doi.org/10.1128/JCM.41.8.3623-3626.2003>.
 34. Sabatelli F, Patel R, Mann PA, Mendrick CA, Norris CC, Hare R, Loebenberg D, Black TA, McNicholas PM. 2006. *In vitro* activities of posaconazole, fluconazole, itraconazole, voriconazole, and amphotericin B against a large collection of clinically important molds and yeasts. Antimicrob Agents Chemother 50:2009–2015. <https://doi.org/10.1128/AAC.00163-06>.
 35. van der Linden JW, Arendrup MC, Warris A, Lagrou K, Pelloux H, Hauser PM, Chrysanthou E, Mellado E, Kidd SE, Tortorano AM, Dannaoui E, Gaustad P, Baddley JW, Uekötter A, Lass-Flörl C, Klimko N, Moore CB, Denning DW, Pasqualotto AC, Kibbler C, Arkan-Akdagli S, Andes D, Meletiadiis J, Naumiuk L, Nucci M, Melchers WJ, Verweij PE. 2015. Prospective multicenter international surveillance of azole resistance in *Aspergillus fumigatus*. Emerg Infect Dis 21:1041–1044. <https://doi.org/10.3201/eid2106.140717>.
 36. Gautier M, Normand AC, Ranque S. 2016. Previously unknown species of *Aspergillus*. Clin Microbiol Infect 22:662–669. <https://doi.org/10.1016/j.cmi.2016.05.013>.
 37. Verweij PE, Zhang J, Debets AJ, Meis JF, van de Veerndonk FL, Schoustra SE, Zwaan BJ, Melchers WJ. 2016. In-host adaptation and acquired triazole resistance in *Aspergillus fumigatus*: a dilemma for clinical management. Lancet Infect Dis 16:e251–e260. [https://doi.org/10.1016/S1473-3099\(16\)30138-4](https://doi.org/10.1016/S1473-3099(16)30138-4).
 38. Verweij PE, Ananda-Rajah M, Andes D, Arendrup MC, Brüggemann RJ, Chowdhary A, Cornely OA, Denning DW, Groll AH, Izumikawa K, Kullberg BJ, Lagrou K, Maertens J, Meis JF, Newton P, Page I, Seyedmousavi S, Sheppard DC, Viscoli C, Warris A, Donnelly JP. 2015. International expert opinion on the management of infection caused by azole-resistant *Aspergillus fumigatus*. Drug Resist Updat 21:30–40. <https://doi.org/10.1016/j.drug.2015.08.001>.
 39. Nedel WL, Pasqualotto AC. 2014. Treatment of infections by cryptic *Aspergillus* species. Mycopathologia 178:441–445. <https://doi.org/10.1007/s11046-014-9811-z>.
 40. Hubka V, Nováková A, Kolařík M, Jurjevič Z, Peterson SW. 2015. Revision of *Aspergillus* section *Flavipedes*: seven new species and proposal of section *Jani* sect. nov. Mycologia 107:169–208. <https://doi.org/10.3852/14-059>.
 41. Peterson SW. 2008. Phylogenetic analysis of *Aspergillus* species using DNA sequences from four loci. Mycologia 100:205–226.
 42. Hubka V, Lyskova P, Frisvad JC, Peterson SW, Skorepova M, Kolarik M. 2014. *Aspergillus pragensis* sp. nov. discovered during molecular re-

- identification of clinical isolates belonging to *Aspergillus* section *Candidi*. *Med Mycol* 52:565–576. <https://doi.org/10.1093/mmy/myu022>.
43. Réblová M, Hubka V, Thureborn O, Lundberg J, Sallstedt T, Wedin M, Ivarsson M. 2016. From the tunnels into the treetops: new lineages of black yeasts from biofilm in the Stockholm metro system and their relatives among ant-associated fungi in the *Chaetothyriales*. *PLoS One* 11:e0163396. <https://doi.org/10.1371/journal.pone.0163396>.
 44. Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* 30:772–780. <https://doi.org/10.1093/molbev/mst010>.
 45. Lanfear R, Calcott B, Ho SY, Guindon S. 2012. PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Mol Biol Evol* 29:1695–1701. <https://doi.org/10.1093/molbev/mss020>.
 46. Nguyen L-T, Schmidt HA, von Haeseler A, Minh BQ. 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol Biol Evol* 32:268–274. <https://doi.org/10.1093/molbev/msu300>.
 47. Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst Biol* 61:539–542. <https://doi.org/10.1093/sysbio/sys029>.
 48. Arendrup MC, Guinea J, Cuenca-Estrella M, Meletiadis J, Mouton JW, Lagrou K, Howard SJ, and the Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID European Committee for Antimicrobial Susceptibility Testing (EUCAST). 2015. EUCAST method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia forming moulds version 9.3. EUCAST, Växjö, Sweden, https://www.aspergillus.org.uk/sites/default/files/pictures/Lab_protocols/EUCAST_E_Def_9_3_Mould_testing_definitive_0.pdf.
 49. Hubka V, Barrs V, Dudová Z, Sklenář F, Kubátová A, Matsuzawa T, Yaguchi T, Horie Y, Nováková A, Frisvad JC, Talbot JJ, Kolařík M. Unravelling species boundaries in the *Aspergillus viridinutans* complex (section *Fumigati*): opportunistic human and animal pathogens capable of inter-specific hybridization. *Persoonia*, in press.