Penicillium-like fungi from clinical samples in the USA and their antifungal susceptibility

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ABSTRACT

Penicillium species are some of the most common fungi observed worldwide, and have an important economic impact as well as being occasional agents of human and animal mycoses. A total of 118 isolates thought to belong to the genus *Penicillium* based on morphological features were obtained from the Fungus Testing Laboratory at the University of Texas Health Science Center in San Antonio (USA). The isolates were studied phenotypically using standard growth conditions. Molecular identification was made using two genetic markers, the internal transcribed spacer (ITS) and a fragment of the β-tubulin gene. In order to assess phylogenetic relationships, maximum likelihood and bayesian inference assessments were used. Antifungal susceptibility testing was determined according to CLSI document M38-A2 for nine antifungals drugs. The isolates were identified within three genera i.e. *Penicillium*, *Talaromyces* and *Rasamsonia*. The most frequent species in our study were *P. rubens*, *P. citrinum* and *T. amestolkiae*. The potent *in vitro* activity of AMB, TRB and of the echinocandins to *Penicillium* and *Talaromyces* species might offer a good therapeutic alternative for the treatment of infections caused by these fungi.

Keywords: *Penicillium, Talaromyces*, *Rasamsonia*, antifungal susceptibility.

INTRODUCTION

Penicillium is one of the largest fungal genera. It comprises some of the most commonly-known filamentous fungi and can be found on numerous substrates, as well as in very diverse habitats (1). Apart from the species included in this genus, many other fungi, such as those included in *Hamigera*, *Paecilomyces*, *Rasamsonia*, *Sagenomella*, *Talaromyces* and *Trichocom*a, also show penicillium-like “little brush” structures. In spite of the morphological similarity of these fungi, recent phylogenetic studies have classified these genera into well-established families, i.e. *Aspergillaceae* (*Hamigera, Penicillium*), *Thermoascaceae* (*Paecilomyces*) and *Trichocomaceae* (*Rasamsonia, Sagenomella*, *Talaromyces, Trichocom*a) (2).

Despite the ubiquity of these fungi in air and in human habitats, their clinical significance is not well understood. Penicillium-like fungi are commonly recovered from clinical samples and in routine hospital air surveys; however they are often discarded as mere contaminants. In addition, their identification to the species level is rarely made in routine laboratories due to the complexity of the phenotypic methods for their *in vitro* study. Further, the high number of species currently accepted in these genera makes this task even more difficult (1, 3). The use of molecular methods, however, does provide a rapid and relatively simple method for the identification of *Penicillium* species, as well as for species in other closely related genera (4, 5).

Partly due to the mentioned aforementioned difficulties, the role of penicillium-like fungi in human pathology has been considered as relatively unimportant. However, one species, *Talaromyces marneffei* (formerly *Penicillium marneffei*), is notable for its clinical relevance as an agent of fatal systemic mycosis, mostly in HIV-

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infected patients, and mainly in southeast Asia, India, and China (6). A few other penicillium-like fungi are seen in the clinical setting, however with a considerably lower incidence. Some species of *Penicillium,* such as *P. chrysogenum*, *P. citrinum*, *P. commune*, *P. decumbens*, *P. piceum*, and *P. purpurogenum* (currently *Talaromyces picesus* and *Talaramoyces purpurogenus*, respectively), have only been reported rarely (7).

Clinical manifestations due to *Penicillium* species include superficial and invasive infections, as well as allergies (8). Infections in humans are mainly related to host immunity (9). There is very little data on animal infections by *Penicillium* species and the few cases reported have been restricted to systemic diseases and fungal osteomyelitis in dogs. *Penicillium brevicompactum*, *P. purpurogenum* and recently *P. canis* have also been reported in fungal infections in dogs (10– 12). Antifungal susceptibility data for clinically available antifungal agents and treatment options for infections caused by *Penicillium* species are also poorly understood, apart from data published for *T. marneffei* (13).

The main goal of the present study was to identify, by molecular means, a large set of clinical and environmental isolates of *Penicillium* and related genera that had been isolated in the USA. The results can provide much-needed information on the diversity of species in that part of the world. Additionally, this study has sought to provide antifungal susceptibility data for the species identified, which will allow more appropriate patient management of these infections.

MATERIALS AND METHODS

Sample collection

A total of 118 isolates identified morphologically as a *Penicillium* sp. were received from the Fungus Testing Laboratory at the University of Texas Health Science Center in San Antonio (UTHSCSA). The isolates were from different locations in the USA and comprised 108 clinical specimens isolated from humans, six from animals, one from a clinical environment, and three that were of unknown origin (Table S1).

Phenotypic characterization

The isolates were subcultured onto malt extract agar (MEA; Difco Laboratories, Detroit, Mich). Phenotypic identification was carried using standard growth conditions described previously (1). For microscopic observation, slides were made with Shear's solution from 7-10 day old cultures. In addition, we evaluated the ability of the isolates to grow at 37ºC.

DNA extraction, amplification and sequencing

The isolates were grown on MEA for 7-14 days at 25ºC prior to DNA extraction. DNA was extracted using the FastDNA® kit protocol (MP Biomedicals, Solon, OH) with the homogenization step using a FastPrep® FP120 cell disrupter (Thermo Savant, Holbrook, NY), according to the manufacturers' instructions. The DNA regions selected for sequencing were those recommended by Visagie (1), for *Penicillium* identification. PCR was performed to amplify the internal transcribed spacer (ITS) of the ribosomal DNA (rDNA), and a fragment of the β-tubulin gene. The primer pairs used were ITS5/ITS4 for the ITS region (14) and the Bt2a/Bt2b for β-tubulin (15).

Single band PCR products were purified and sequenced at Macrogen Corp. Europe104 (Amsterdam, the Netherlands) with a 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). Sequence assembly and editing were performed using SeqMan v. 7.0.0 (DNASTAR, Madison, WI). The nucleotide sequences obtained

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were submitted to GenBank under the accession numbers LT558856- LT558973 for ITS and LT558974- LT559090 for β-tubulin (Table S1).

Phylogenetic reconstructions

Preliminary identification of the isolates to the genus level was determined with the analysis of ITS sequences, using the BLAST algorithm implemented in the GenBank, CBS-KNAW, and MycoBank databases. Isolates were identified to the species level using the ITS and β-tubulin sequences. Multiple sequence alignments were performed for each locus in MEGA v 6.0 software (16), using the CLUSTALW algorithm (17), refined with MUSCLE (18), and manually adjusted using the same software platform.

Phylogenetic analyses were made with the individual loci and combined genes using maximum-likelihood (ML) in MEGA v. 6.0 (16) and Bayesian inference (BI) under MrBayes version 3.1.2 (19). For ML, support for internal branches was assessed by 1,000 ML bootstrapped pseudoreplicates of data. A bootstrap support (bs) ≥ 70 was considered significant. The phylogenetic reconstruction by BI was performed using five million Markov chain Monte Carlo (MCMC) generations, with two runs (one cold chain and three heated chains) and samples were stored every 1,000 generations. The 50% majority-rule consensus tree and posterior probability values (pp) were calculated after discarding the first 25% of the samples. A pp value ≥0.95 was considered significant. The best substitution model for all gene matrices was estimated using jModelTest v.2.1.3 (20, 21).

Antifungal susceptibility testing

Antifungal susceptibility of the isolates was determined according to the Clinical and Laboratory Standards Institute (CLSI) broth microdilution M38-A2 method for

filamentous fungi (22). The *in vitro* activities of amphotericin B (AMB), voriconazole (VRC), itraconazole (ITC), posaconazole (PSC), terbinafine (TRB), anidulafungin (AFG), caspofungin (CFG), micafungin (MFG) and 5-fluorocytosine (5FC) were determined for those species with five or more isolates. The minimum inhibitory concentration (MIC) was defined as the lowest concentration to inhibit 100% of growth on visual inspection for AMB, ITC, PSC and VRC and to reduce growth by 80% for TRB compared to the drug-free control well. The minimal effective concentration (MEC) was defined as the lowest concentration to produce short, stubby, abnormally branched hyphae for the echinocandins. Both parameters, MIC and MEC, were determined at 48 h

Candida krusei ATCC 6258, *Candida parapsilosis* ATCC 22019, *Paecilomyces variotii* ATCC MYA-3630 and *Aspergillus fumigatus* ATCC MYA-3626 were used as quality control strains for all tests.

RESULTS

Based on the analysis of the ITS region, we discovered that the 118 isolates investigated were nested in 85 belonging to *Penicillium* (n=85), *Talaromyces* (n=31) or *Rasamsonia* (n=2). Identification of the isolates at the species level through phylogenetic analysis with the combination of ITS and β-tubulin sequences is summarized in Table 1.

We carried out a phylogenetic study for each of the three genera involved. The first one (Figure S1) was to identify *Penicillium* isolates by grouping them into their respective sections. The aligned data set was 919 bp long (ITS 521 bp and β-tubulin 398 bp) and Kimura's two parameter (K2) with gamma distribution (+G) was

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the model selected for each fragment. This analysis showed that our isolates were distributed into 23 species belonging to the following ten sections: *Chrysogena* (n=*28*), *Citrina* (*n=17*), *Fasciculata* (*n*=11), *Lanata-Divaricata* (*n*=11), *Aspergilloides* (n=7)*, Exilicaulis* (n=4), *Roquefortorum* (n=3), *Brevicompacta* (n=*2*)*, Penicillium* (n=1), and *Sclerotiora* (n=1). The most frequently identified taxa were *P. rubens* (22.4%, n=19, Section *Chrysogena*) and *P. citrinum* (16.5%, n=14, Section *Citrina*). Additionally, within of section *Citrina,* seven *Penicillium* sp. isolates could not be identified at species level.

A second phylogenetic reconstruction (Figure S2) was performed to identify the *Talaromyces* isolates. The aligned data set was 847 bp long (ITS 474 bp and β-tubulin 373 bp) and the model selected was Tamura three parameter (T92) with gamma-distributed rates and the presence of invariant sites (G+I) for ITS and K2 +G+I for β-tubulin. In this genus, ten species were identified belonging to four sections, i.e., *Talaromyces* (n=25), *Tachyspermi* (n=3), *Islandici* (n=2) and *Helici* (n=1). The most prevalent species were *T. amestolkiae* (22.6%, n=7) and *T. purpurogenus* (16.1%, n=5), both in section *Talaromyces*. A total of six isolates could not be identified at a species level: *Talaromyces* sp. I (n=1; section *Talaromyces*), *Talaromyces* sp. II (n=2; section *Talaromyces), Talaromyces* sp. III (n=1; section *Talaromyces), Talaromyces* sp. IV (n=1; section *Helici*), *Talaromyces* sp. V (n=1; section *Islandici*) and *Talaromyces* sp. VI (n=1; section *Tachyspermi*).

The third phylogenetic analysis (Figure S3) was carried out to identify the *Rasamsonia* isolates. The aligned data set was 1078 bp long (ITS 599 bp, and β-tubulin 479 bp) and the selected models for each fragment were T92 and K2 with

uniform rates for ITS and β-tubulin, respectively. The isolates were identified as *R. argillacea* and *R. eburnea*.

The 118 isolates were mainly from the respiratory tract (72.9%), usually from human bronchoalveolar lavage (BAL) (Table S1). We carried out the antifungal susceptibility testing of the most frequent species, i.e, a total of 51 isolates (39 *Penicillium* and 12 *Talaromyces*) representing seven species (Tables 2 and 3). Overall, TRB and the echinocandins showed the best *in vitro* activity against *Penicilium* species, with a mode of <0.03 µg/mL for TRB and of 0.06 µg/mL for CFG and AFG, and 0.125 µg/mL for MFG. Amphotericin B showed intermediate antifungal activity, with overall mode of 2 µg/mL, while the azoles showed variable activity, with wide MIC ranges and modes of 0.5 µg/mL for PSC and ITC, and 2 µg/mL for VRC. The highest MIC values were observed for 5FC (Table 2). *Talaromyces* species were similarly active *in vitro* to TRB, echinocandins and AMB, while 5FC showed good *in vitro* activity compared to *Penicillium* species with a mode of 0.125 µg/mL. By contrast, the azoles showed poor *in vitro* activity with wide MIC ranges and mode of >16 µg/mL to PSC, VRC and ITC (Table 3).

The results from growth at 37ºC (Table 1) showed that 71.7% of the *Penicillium* isolates (n=61) were able to grow at this temperature. All the isolates of both sections *Chrysogena* (*n*=28) and *Lanata-Divaricata* (n=11) grew at 37 ºC. Whereas, in section *Citrina*, only the isolates identified as *P. citrinum* managed to grow at this physiologically relevant temperature. On the other hand, practically all isolates of the genus *Talaromyces* (99.6%, n=29) grew at 37ºC, as well as the two isolates of *Rasamsonia* (Table 1).

DISCUSSION

Despite the uncertainty of the true role that penicillium-like fungi plays in human pathology, there are several reports that seem to involve these fungi in the clinical setting, especially for isolates from respiratory samples (23, 24). However, to our knowledge, the species diversity of a large collection of penicillium-like fungi from clinical origin has never been explored. Thus, this is the first study to investigate more than one hundred isolates from clinical sources and to demonstrate, by using the combined sequence analysis of the ITS region and β-tubulin gene, that three different genera of penicillium-like fungi (i.e., *Penicillium, Talaromyces* and *Rasamsonia*) are, in fact, associated with these types of samples. As expected, *Penicillium* was the most common (72%).

The most frequently identified species within *Penicillium* were *P. citrinum* and *P. rubens. Penicillium citrinum* has been reported as a human opportunistic pathogen responsible for keratitis, cutaneous infections and pneumonia (25- 28).

Curiously, to date, *P. rubens* has not shown any link to clinical isolates, although it is a recently resurrected species, closely related to *P. chrysogenum* (29). By contrast, *P. chrysogenum* has already been reported as a human pathogen associated with cutaneous and invasive infections (8, 30, 31). Twenty eight of our isolates were classified within section *Chrysogena,* 19 identified as *P. rubens*, two as *P. chrysogenum* and seven isolates very similar to those, but which will require additional phylogentic markers to distinguish them properly (29). Although we were not able to demonstrate the pathogenic role of *P. rubens* and *P. chrysogenum*, the high number of strains of this species recovered and their ability to grow at 37ºC highlight the clinical importance of these species.

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Other *Penicillium* species found in our study were *P. glabrum* and *P. oxalicum*, which were the most frequent species after *P. rubens* and *P. citrinum*. While *P. glabrum* has not been associated with human infections, Chowdhary (32) reported three cases of invasive infections by *P. oxalicum* in patients with acute myeloid leukemia, diabetes mellitus, and chronic obstructive pulmonary disease. The lung was theorized as the portal of entry for this pathogen in these three cases.

Several reports have recognized *P. decumbens* as the cause of a disseminated infection, a peri-operative paravertebral infection and a fungus ball (7). However, only one isolate of *P. decumbens* was identified in our study, which represents another rare species in the clinical setting (32). Some other *Penicillium*, many of them represented by only one isolate in our study, such as *P. rubefaciens*, *P. brasilianum*, *P. singorense* and *P. rudallense,* have not been recognized previously from clinical specimens but their ability to grow at 37ºC suggests a potential pathogenicity.

It is relevant that a considerable number of our isolates were identified as *Talaromyces*. Apart from *T. marneffei*, which is the most clinically important member, this genus contains some opportunistic species of clinical importance such as *T. amestolkiae, T. indigoticus, T. piceus, T. purpurogenus, T. radicus, T. ruber, T. rugulosus, T. stollii* and *T. verruculosus.* Most of these species were part of the genus *Penicillium* (3, 33). Nearly 80 % of our isolates identified as *Talaromyces* belong to section *Talaromyces*, which is the only section that includes both animal and human pathogenic species (33). *Talaromyces amestolkiae* and *T. purpurogenus* were the species most frequent identified among our isolates. These, together with *T. ruber* and *T. stollii*, were recovered from pulmonary and invasive infections in humans (7,

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9) and animals (8, 12). The production of a red diffusible pigment by the colonies of *T. purpurogenum* is a feature shared with *T. marneffei*, which can lead to misidentification of those two species in diagnostic laboratories. Although the former can grow at 37 ºC, it is unable to develop a yeast morphology like *T. marneffei* can (9).

We also identified two isolates belonging to *Rasamsonia,* one as *R. argillacea* and the other as *R. eburnea* (formerly *Talaromyces eburneus*). Recently, infections by isolates within this genus appear to have increased in humans and animals, and are considered as emerging pathogens (34- 36). In 2011, nine cases of invasive infections by *Rasamsonia argillacea* were reported in patients with chronic granulomatous disease (37, 38), and more recently in a patient with graft-versus-host disease (39). Houbraken, et al. (35) identified clinical isolates of *R. eburnea* from blood cultures, sputum and peritoneal dialysis fluid from a patient with peritonitis. *Rasamsonia argillacea* and *R. eburnea* are phylogenetically close and have similar phenotypic characteristics, except that *R. eburnea* shows a blackish brown reverse (40).

Several cases have been reported where the respiratory tract was the portal of entry of infections by penicillium-like fungi, with or without systemic dissemination. Although the majority of isolates in this study were obtained from respiratory specimens, it was not possible to establish the true pathogenic role of the identified species because of the nature of the samples and the absence of clinical data.

In vitro antifungal susceptibility profiles of penicillium-like fungi species other than *T. marneffei* are currently based on very few studies and are mainly taken from case reports that have shown variable results (8, 27, 32). Our results show that TRB

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and the echinocandins are highly active *in vitro* against *Penicillium* and *Talaromyces*. However, these antifungals are not widely used for treating invasive fungal infections by these fungi (41). Terbinafine was chosen as a good alternative for long-term maintenance therapy against one isolate from a dog with osteomyelitis (11). In the present study, AMB also had intermediate antifungal activity, agreeing with previous studies (42, 43); however, the clinical experience reported in two cases of infections by species within this group revealed that the patients did not responded to this drug (8). Our susceptibility results show that the azoles have variable activity against *Penicillium* species, and high MICs for *Talaromyces*. In fact, ITC has been used as prophylactic treatment in infections by *T. marneffei* (42). Chowdhary et al. (32) reported resistance to VRC in three cases of invasive infections by *P. oxalicum*, where the successful alternative treatment was PSC. We observed intermediate MIC values for VRC in our isolates of *P. oxalicum* while the *P. citrinum* isolates showed resistance to this drug. This confirms the observations of Mok et al., (27), who reported high MIC values for the azoles against one isolate of *P. citrinum* from an acute leukemia patient with pneumonia and pericarditis.

In conclusion, although human and animal infections caused by penicillium-like fungi are infrequent, this study reveals that a relative wide range of species, all able to grow at 37ºC, should be taken into account in the diagnosis of such infections. Identification at the species-level remains difficult on the grounds that various genera share similar morphological characteristics. This supports the relevance of using DNA sequence data to identify them. More data are needed from both *in vitro* susceptibility studies and clinical outcomes in order to determine an effective treatment for infections caused by penicilium-like fungi.

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452 **Table 1.** Molecular identification and growth at 37ºC of the isolates included in the study.

Genus (nº. of isolates)	Species	Section	nº of isolates	Growth at 37 °C (mm/7d)
Penicillium (85)	*P. rubens*	*Chrysogena*	19	19
	P. citrinum	*Citrina*	14	14
	Penicillium sp.	*Chrysogena*	7	7
	P. oxalicum	*Lanata-Divaricata*	7	7
	P. glabrum	*Aspergilloides*	5	4
	P. crustosum	*Fasciculata*	4	0
	P. polonicum	*Fasciculata*	4	1
	P. roqueforti	*Roquefortorum*	3	0
	P. chrysogenum	*Chrysogena*	2	2
	P. citreonigrum	*Exilicaulis*	2	0
	P. rolfsii	*Lanata-Divaricata*	2	2
	P. brevicompactum	*Brevicompacta*	2	0
	P. sumatrense	*Citrina*	1	0
	P. pancosmium	*Citrina*	1	0
	P. roseopurpureum	*Citrina*	1	0
	P. decumbens	*Exilicaulis*	1	1
	P. rubefaciens	*Exilicaulis*	1	1
	P. allii	*Fasciculata*	1	0
	P. echinulatum	*Fasciculata*	1	0
	P. palitans	*Fasciculata*	1	0
	P. brasilianum	*Lanata-Divaricata*	1	1
	P. singorense	*Lanata-Divaricata*	1	1
	P. adametzioides	*Sclerotiora*	1	0
	P. coprophilum	*Penicillium*	1	0
	P. frequentans	*Aspergilloides*	1	0
	P. rudallense	*Aspergilloides*	1	1
Talaromyces (31)	*T. amestolkiae*	*Talaromyces*	7	7
	T. purpureogenus	*Talaromyces*	5	5
	T. pinophilus	*Talaromyces*	3	3
	T. aurantiacus	*Talaromyces*	2	2
	T. ruber	*Talaromyces*	2	2
	Talaromyces spp. I	*Talaromyces*	2	2
	Talaromyces spp. II	*Talaromyces*	1	1
	Talaromyces spp. III	*Talaromyces*	1	0
	T. cnidii	*Talaromyces*	1	1
	T. funiculosus	*Talaromyces*	1	1
	Talaromyces spp. IV	*Helici*	1	1
	T. columbinus	*Islandici*	1	1
	Talaromyces spp. V	*Islandici*	1	1
	T. atroroseus	*Tachyspermi*	1	1
	T. diversus	*Tachyspermi*	1	1
	Talaromyces spp. VI	*Tachyspermi*	1	0

Rasamsonia (2)	*R. argillacea*	-	1	1
	R. eburnea	-	1	1

453

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454 **Table 2.** Results of *in vitro* antifungal susceptibility testing of 39 isolates of *Penicillium*
455 species.
456

Species (no. of isolates tested)	Antifungal*	No. of isolates with MIC (µg/mL) of:										
		≤ 0,03	0,03	0,06	0,125	0,25	0,5	1	2	4	8	16	> 16
P. citrinum (n=10)	CFG		2	7	1								
	AFG		4	5	1								
	MFG		1	1	7	1							
	TRB	3	7										
	PSC				1			7	2				
	VRC												10
	ITC						1	4	1	1			3
	AMB							3	7				
	5FC									2	4	3	1
P. rubens (n=10)	CFG		1	2	4	3							
	AFG		1	4	1	4							
	MFG		1	2	5	2							
	TRB	8	1	1									
	PSC				2	2	6						
	VRC					1		2	4	1			2
	ITC			2		1	7						
	AMB				1		1	4	4				
	5FC							1	2	4	2		1
Penicillium sp. (n=7)	CFG			1	6								
	AFG		1	1	1	4							
	MFG				3	3		1					
	TRB	3	4										
	PSC				1	1	4	1					
	VRC					1	1	2	3				
	ITC				2	2	2	1					
	AMB							1	6				
	5FC												7
P. oxalicum (n=7)	CFG			2	1	2			1	1			
	AFG		1	2	1		1			2			
	MFG				2	2		1	1	1			
	TRB	5	2										
	PSC					4	2	1					
	VRC								7				
	ITC				1	1		1	4				
	AMB					2	4	1					
	5FC							1		1	4		1
P. Glabrum (n=5)	CFG		5										
	AFG		4		1								
	MFG		3	2									

	TRB	2		3								
	PSC				2	1	2					
	VRC							1	3	1		
	ITC			1	1		1	2				
	AMB			1	2	2						
	5FC							3	1	1		

*CFG, caspofungin; AFG, anidulafungin; MFG, micafungin; TRB, terbinafine; PSC, posaconazole, VRC, voriconazole; ITC, itraconazole; AMB, amphotericin B and 5FC, flucytosine.

Table 3. Results of *in vitro* antifungal susceptibility testing of 12 isolates of *Talaromyces* species.

Species (no. of isolates tested)	Antifungal*	No. of isolates with MIC (µg/mL) of:										
		≤ 0,03	0,03	0,06	0,125	0,25	0,5	1	2	4	8	16	> 16
T. amestolkiae (7)	CFG			2	3	1		1					
	AFG		1	3	3								
	MFG			3	2	2							
	TRB	6	1										
	PSC						2		1				4
	VRC									1			6
	ITC									1			6
	AMB				1	2	2	2					
	5FC			2	3		1		1				
T. purpurogenus (5)	CFG					1				1	1	2	
	AFG			1	3							1	
	MFG			2	1	2							
	TRB	1	2	1								1	
	PSC								1	1		1	2
	VRC								1	2	2		
	ITC												5
	AMB								3	2			
	5FC			3	2								

*CFG, caspofungin; AFG, anidulafungin; MFG, micafungin; TRB, terbinafine; PSC, posaconazole, VRC, voriconazole; ITC, itraconazole; AMB, amphotericin B and 5FC, flucytosine.