ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 2007, p. 748–751 Vol. 51, No. 2
0066-4804/07/$08.00+0 doi:10.1128/AAC.01177-06

Prevalence and Susceptibility Testing of New Species of *Pseudallescheria* and *Scedosporium* in a Collection of Clinical Mold Isolates▽

Ana Alastruey-Izquierdo, Manuel Cuenca-Estrella, Araceli Monzón, and Juan L. Rodriguez-Tudela*

Servicio de Micología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid, Spain

Received 21 September 2006/Returned for modification 21 October 2006/Accepted 4 November 2006

The prevalence of new species of *Pseudallescheria* and *Scedosporium* in a collection of 46 clinical isolates was analyzed. Strain identification was done by morphological and molecular methods. Four *Scedosporium aurantiacum* isolates were detected among the panel of clinical strains. The susceptibility profile of *S. aurantiacum* was similar to that of *Scedosporium apiospermum*.

Pseudallescheria boydii is a pathogen able to cause asymptomatic colonization and localized and disseminated infections (11).

Recently, it has been demonstrated that high genetic variation exists in the *P. boydii* species complex (2, 4, 8). Two new species, *Pseudallescheria minutispora* and *Scedosporium aurantiacum*, are phylogenetically and morphologically separated from *P. boydii*. In addition, *P. angusta*, *P. ellipsoidea*, and *P. fusoidea* seem to present genetic differences and could be proposed as new species of *Pseudallescheria* (4).

We have analyzed the prevalence of these new species and their antifungal susceptibility profiles in a collection of clinical isolates of *P. boydii*.

Strains. A total of 46 clinical isolates of *P. boydii* and *S. apiospermum* were included in this study. Twenty-four strains were isolated from respiratory sites, five from biopsies, six from ear samples, five from skin, four from ocular samples, one from blood culture, and one from an abscess.

Table 1 displays the identification of the 42 sequences obtained from the GenBank database that were used as controls.

Morphological identification. All isolates were identified by conventional methods (3).

PCR and DNA sequencing of the internal transcribed spacer (ITS) region. Molds were cultured in GYEP medium (0.3% yeast extract, 1% peptone [Difco, Madrid, Spain], 2% glucose [Sigma Aldrich Quimica, Madrid, Spain]) for 24 to 48 h at 30°C. Genomic DNA was isolated using a previously described extraction procedure (5).

DNA segments comprising the ITS1 and ITS2 regions were amplified with primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (1) in a GeneAmp 9700 PCR system (Applied Biosystems) (10). The reaction products were analyzed in a 0.8% agarose gel.

Sequencing reactions were done with 2 μl of reaction mixture from a sequencing kit (BigDye terminator cycle sequencing kit, Ready Reaction mixture; Applied Biosystems), 1 μM of the primers (ITS1 and ITS4), and 3 μl of the PCR product in a final volume of 10 μl.

Sequence analysis. Sequences were assembled and edited using the SeqMan II and EditSeq software packages (Lasergene; DNAstar, Inc., Madison, WI). Sequence analysis was performed by comparison of the DNA sequences with

TABLE 1. *Scedosporium* reference strains used for comparison of ITS sequences and their GenBank accession numbers

Species	Strain identification	GenBank accession no.
Pseudallescheria angusta		AJ888414
Pseudallescheria angusta		AJ888413
Pseudallescheria ellipsoidea	CBS418,73	AJ888426
Pseudallescheria ellipsoidea		AJ888427
Pseudallescheria fusoidea	CBS106,53	AJ888428
Pseudallescheria fusoidea		AJ888429
Pseudallescheria minutispora		AJ888424
Pseudallescheria minutispora		AY228119
Pseudallescheria minutispora		AJ888384
Scedosporium apiospermum		AJ888443
Scedosporium apiospermum		AJ888392
Scedosporium apiospermum		AF181558
Scedosporium apiospermum		AJ888385
Scedosporium apiospermum		AJ888438
Scedosporium apiospermum		AJ888391
Scedosporium apiospermum		AY213683
Scedosporium apiospermum	CBS 10854	AY228112
Scedosporium apiospermum		AJ888400
Scedosporium apiospermum		AY217658
Scedosporium apiospermum		AY228123
Scedosporium apiospermum		AY939802
Scedosporium apiospermum		AF455484
Scedosporium apiospermum	CBS 10122	AY213680
Scedosporium apiospermum		AY213681
Scedosporium apiospermum	CBS 59190	AY228118
Scedosporium apiospermum		AY228122
Scedosporium apiospermum		AY213682
Scedosporium apiospermum		AJ888398
Scedosporium apiospermum		AJ888436
Scedosporium apiospermum		AJ888386
Scedosporium apiospermum		AJ888387
Scedosporium apiospermum		AJ888394
Scedosporium apiospermum		AJ888397
Scedosporium apiospermum		AJ888405
Scedosporium apiospermum		AJ888410
Scedosporium apiospermum		AJ888411
Scedosporium apiospermum	CBS101,22	AJ888435
Scedosporium apiospermum		AJ888437
Scedosporium aurantiacum		AJ888432
Scedosporium aurantiacum		AJ888439
Scedosporium aurantiacum		AJ888440
Scedosporium aurantiacum		AJ888441

* Corresponding author. Mailing address: Servicio de Micología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Ctra. Majadahonda-Pozuelo Km 2, 28220 Majadahonda (Madrid), Spain. Phone: 34-91-8223661. Fax: 34-91-5097966. E-mail: juanl.rodriguez-tudela@isciii.es.

▽ Published ahead of print on 13 November 2006.

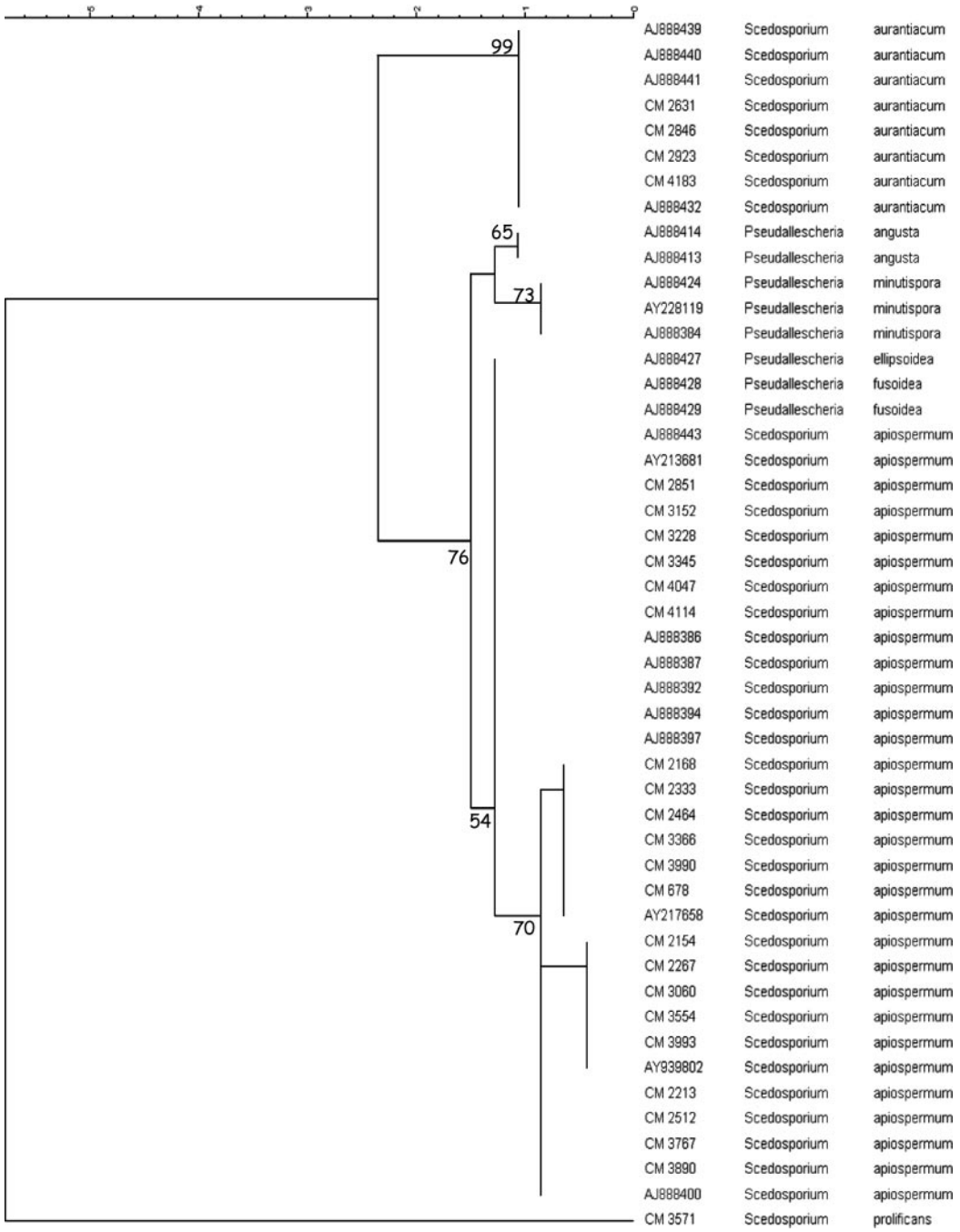

FIG. 1. Phylogenetic tree of the subset of isolates included in the study obtained by using maximum parsimony phylogenetic analyses and 2,000 bootstrap simulations based on ITS sequences. *Scedosporium prolificans* CNM-CM-3751 was used as the outgroup to root the tree.

42 ITS sequences of *Scedosporium* and *Pseudallescheria* strains obtained from the GenBank database (http://www.ncbi.nih.gov/GenBank/). Full information about these strains is displayed in Table 1.

Phylogenetic analysis. All phylogenetic analyses were conducted with Fingerprinting II Informatix software, version 3.0 (Bio-Rad Laboratories, Madrid, Spain). The methodology used was maximum parsimony clustering. Phylogram stability was assessed by parsimony bootstrapping with 2,000 simulations. The ITS sequence of *Scedosporium prolificans* CNM-CM-3571 (Mold Collection of the Spanish National Center for Microbiology) was used as the outgroup.

Antifungal susceptibility testing. Microdilution testing was performed following the CLSI (formerly NCCLS) reference method (6) with the following minor modifications. (i) RPMI 1640 was supplemented with glucose to reach a 2% concentration. (ii) Inoculum size was between 1×10^5 to 5×10^5 CFU/ml. Inoculum preparations were performed by means of

TABLE 2. Antifungal susceptibility results for clinical isolates of *Scedosporium aurantiacum* and geometric mean and range of the 42 *Scedosporium apiospermum* isolates

Isolate	Strain no.	Sample	MIC (mg/liter)		
			Amphotericin B	Itraconazole	Voriconazole
S. aurantiacum	CNM-CM-2923	Corneal	8	16	16
	CNM-CM-2631	Sputum	4	2	0.5
	CNM-CM-2846	Ear swab	16	8	1
	CNM-CM-4183	Bronchial aspirate	8	16	0.5
Geometric mean			6.96	6.06	1.15
S. apiospermum					
Geometric mean			6.21	2.62	0.73
Range			0.25–32	0.25–16	0.125–16

counting spores in a hematocytometer (1, 7, 9). *Aspergillus fumigatus* ATCC 2004305 and *Aspergillus flavus* ATCC 2004304 were used as quality control strains (6).

The antifungal agents used in the study were amphotericin B (range, 16 to 0.03 mg/liter) (Sigma Aldrich Química), itraconazole (range, 8 to 0.015 mg/liter) (Janssen S.A., Madrid, Spain), and voriconazole (range, 8 to 0.015 mg/liter) (Pfizer S.A.). The endpoint was the antifungal concentration that produced a complete inhibition of visual growth at 48 h.

Morphological identification. All strains were identified as *Scedosporium apiospermum* by conventional methods (3). We were not able to identify by morphological characteristics the new proposed species (4, 8).

Molecular identification. Figure 1 shows the phylogenetic analysis by means of maximum parsimony of a subset of the strains included in the study. Among 46 clinical strains analyzed, four *S. aurantiacum* strains were identified with a bootstrap value of ≥99. They were isolated from sputum, bronchial aspirate, a corneal sample, and an ear swab. In this collection of clinical isolates, we did not found any strain of *P. minutispora* or *P. angusta*, but the sequences obtained from GenBank were supported by bootstrap values of 73 and 65, respectively. On the other hand, the analyses of ITS sequences indicate that *Pseudallescheria ellipsoidea* and *P. fusoidea* are indistinguishable from *S. apiospermum* isolates (Fig. 1).

Antifungal susceptibility testing. The MICs of antifungal agents for the collection of clinical isolates are shown in Table 2. They are sorted by species identification by means of ITS sequencing.

It has always been suspected that *Pseudallescheria boydii* was a complex of species. Hitherto, morphological analyses of *Pseudallescheria boydii* isolates did not allow for a clear separation among different species. However, the analysis of DNA sequences has permitted the proposal of new species from *Pseudallescheria*, such as *S. aurantiacum*, *P. minutispora*, *P. angusta*, *P. ellipsoidea*, and *P. fusoidea* (4, 8).

We examined the prevalence of these new species in a collection of clinical isolates. ITS sequence analyses revealed the presence of four *S. aurantiacum* strains, obtained from an ocular sample, an ear swab, bronchial aspirate, and sputum, and no strains of *P. minutispora*. Although Gilgado et al. (4) have defined morphological characteristics to identify *S. aurantiacum* and *P. minutispora*, great experience in classical taxonomy is required to perform this task. Therefore, it is not expected that many clinical microbiology laboratories are able to identify those new species unless they use molecular methodology. Regarding susceptibility to antifungal drugs, *S. aurantiacum* seems slightly more resistant than *S. apiospermum* to amphotericin B and itraconazole, although a higher number of isolates should be analyzed before any conclusion is drawn (Table 2).

Because little is know about the prevalence of these new species and therefore there are no studies regarding epidemiology, pattern of disease, risk factors, antifungal susceptibility testing, etc., we strongly recommend sending all strains of *Scedosporium* species involved in human infections to reference laboratories where those isolates can be properly identified to the species level and antifungal susceptibility testing can be performed. In this way, the importance of these new species can be ascertained. Meanwhile, and especially for clinical use, it would be better to maintain the use of the *S. apiospermum* name. From a practical point of view and for clinical microbiology laboratories, we suggest performing antifungal susceptibility testing rather than applying molecular methods for the identification of these species.

Ana Alastruey-Izquierdo has a predoctoral fellowship from Fondo de Investigaciones Sanitarias (grant FI05/00856). This work was supported in part by research project PY05/32 from the Instituto de Salud Carlos III.

REFERENCES

1. **Aberkane, A., M. Cuenca-Estrella, A. Gomez-Lopez, E. Petrikkou, E. Mellado, A. Monzon, and J. L. Rodriguez-Tudela.** 2002. Comparative evaluation of two different methods of inoculum preparation for antifungal susceptibility testing of filamentous fungi. J. Antimicrob. Chemother. **50:**719–722.
2. **Defontaine, A., R. Zouhair, B. Cimon, J. Carrere, E. Bailly, F. Symoens, M. Diouri, J. N. Hallet, and J. P. Bouchara.** 2002. Genotyping study of *Scedosporium apiospermum* isolates from patients with cystic fibrosis. J. Clin. Microbiol. **40:**2108–2114.
3. **de Hoog, G. S., J. Guarro, J. Gené, and M. J. Figueras.** 1995. Hyphomycetes, p. 301–302 and 899–901. *In* G. S. de Hoog and J. Guarro (ed.), Atlas of clinical fungi. Universitat Rovira i Virgili, Reus, Spain.
4. **Gilgado, F., J. Cano, J. Gene, and J. Guarro.** 2005. Molecular phylogeny of the *Pseudallescheria boydii* species complex: proposal of two new species. J. Clin. Microbiol. **43:**4930–4942.
5. **Holden, D. W.** 1994. DNA mini prep method for *Aspergillus fumigatus* (and other filamentous fungi), p. 3–4. *In* B. Maresca and G. S. Kobayashi (ed.), Molecular biology of pathogenic fungi, a laboratory manual. Telos Press, New York, NY.
6. **National Committee for Clinical Laboratory Standards.** 2002. Reference

method for broth dilution antifungal susceptibility. Testing of filamentous fungi: approved standard document M38-A. Reference method for broth dilution antifungal susceptibility 22. NCCLS, Wayne, Pa.
7. **Petrikkou, E., J. L. Rodriguez-Tudela, M. Cuenca-Estrella, A. Gomez, A. Molleja, and E. Mellado.** 2001. Inoculum standardization for antifungal susceptibility testing of filamentous fungi pathogenic for humans. J. Clin. Microbiol. **39:**1345–1347.
8. **Rainer, J., G. S. de Hoog, M. Wedde, Y. Graser, and S. Gilges.** 2000. Molecular variability of *Pseudallescheria boydii*, a neurotropic opportunist. J. Clin. Microbiol. **38:**3267–3273.
9. **Rodriguez-Tudela, J. L., E. Chryssanthou, E. Petrikkou, J. Mosquera, D. W. Denning, and M. Cuenca-Estrella.** 2003. Interlaboratory evaluation of hematocytometer method of inoculum preparation for testing antifungal susceptibilities of filamentous fungi. J. Clin. Microbiol. **41:**5236–5237.
10. **Rodriguez-Tudela, J. L., T. M. Diaz-Guerra, E. Mellado, V. Cano, C. Tapia, A. Perkins, A. Gomez-Lopez, L. Rodero, and M. Cuenca-Estrella.** 2005. Susceptibility patterns and molecular identification of *Trichosporon* species. Antimicrob. Agents Chemother. **49:**4026–4034.
11. **Steinbach, W. J., and J. R. Perfect.** 2003. Scedosporium species infections and treatments. J. Chemother. **15**(Suppl. 2)**:**16–27.