

Antifungal susceptibility profile of clinical *Alternaria* spp. identified by molecular methods

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Received 28 April 2011; returned 16 June 2011; revised 2 August 2011; accepted 15 August 2011

Objectives: To analyse the susceptibility pattern of a collection of *Alternaria* spp. clinical isolates.

Methods: The antifungal susceptibilities of 35 isolates identified by means of sequencing the internal transcribed spacer region of rDNA were analysed by European Committee on Antimicrobial Susceptibility Testing (EUCAST) methodology.

Results and conclusions: No clear differences among the activity of antifungals against *Alternaria alternata* and *Alternaria infectoria* were detected, except for echinocandins.

Keywords: *Alternaria* spp., susceptibility testing, molecular identification

Introduction

Alternaria is a dematiaceous ascomycete with a worldwide distribution. The genus comprises a large number of species, mostly saprophytes or plant pathogens. It can be found in soil, air, and it has also been described in normal human and animal skin and conjunctiva. In humans, it is frequently associated with allergic respiratory diseases and is increasingly associated with opportunistic infections in immunocompromised hosts. Thus, it has been described as the causative agent of keratitis, peritonitis, osteomyelitis, sinusitis, and cutaneous and subcutaneous infections associated with a previous trauma.^{1,2}

Alternaria alternata and *Alternaria infectoria* are the species most frequently encountered in clinical samples,² although other species, such as *Alternaria tritici*, *Alternaria tenuissima*, *Alternaria chlamydospora*, *Alternaria longipes* and *Alternaria dianthicola*, have also been reported. The real prevalence of these species in clinical samples is unknown, since a full identification to the species level was not performed in most of the cases described in the literature. The lack of sporulation in routine media for most *Alternaria* spp. strains makes its identification by classical morphological methods impossible. The use of molecular techniques [sequencing of the internal transcribed spacer (ITS) region of rDNA] has been shown to be a useful method for the identification of *Alternaria* spp. using sequences of reference strains as comparison.³

In general, previous *in vitro* studies report that *Alternaria* shows a good response to conventional antifungal drugs.^{2,4} However, the information regarding the antifungal susceptibility profile of *Alternaria* spp. is very limited, especially because, as stated above, most of the reports published did not use

molecular methods to identify isolates to the species level. In this work, we report the antifungal susceptibilities of 35 isolates identified by means of sequencing the ITS region of rDNA.

Materials and methods

Strains

A total of 34 clinical isolates and 1 environmental isolate of *Alternaria* spp. were included in the study. Each isolate was obtained from a different patient. Ten strains were isolated from biopsies, 8 from respiratory sites, 8 from ocular samples, 6 from cutaneous sites, 1 from an ear exudate, 1 from an abscess and 1 had an environmental origin. The strains were identified by morphological and molecular methods (ITS sequencing). *A. infectoria* CBS210.86^T=EGS27-193=AF081456 and *A. alternata* CBS916.96^T=EGS34-016=AF071346 type strains were used as reference sequences for comparison purposes.

Antifungal susceptibility testing

Microdilution testing was performed following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standard methodology.⁵ *Aspergillus fumigatus* ATCC 2004305 and *Aspergillus flavus* ATCC 2004304 were used as quality control strains. The antifungal agents used in the study were amphotericin B (Sigma–Aldrich Quimica, Madrid, Spain), itraconazole (Janssen Pharmaceutica, Madrid, Spain), voriconazole (Pfizer S.A., Madrid, Spain), ravuconazole (Bristol-Myers Squibb, Princeton, NJ, USA), posaconazole (Schering-Plough Research Institute, Kenilworth, NJ, USA), terbinafine (Novartis, Basel, Switzerland), caspofungin (Merck & Co., Inc., Rahway, NJ, USA), micafungin (Astellas Pharma Inc., Tokyo, Japan) and anidulafungin (Pfizer SA). The final concentrations tested ranged from 16 to 0.03 mg/L for amphotericin B, terbinafine, caspofungin, micafungin and anidulafungin, and from 8 to 0.015 mg/L for

Table 1. Antifungal susceptibility results of clinical isolates of *Alternaria* spp.

Species (no. of isolates)		MIC (mg/L)								
		AMB	ITC	VRC	RCZ	POS	TRB	CAS	MIC	AND
<i>A. alternata</i> (19)	GM	0.36	1.79	3.56	3.69	1.23	7.44	1.56	0.67	0.18
	range	0.03–32	0.125–16	0.5–16	0.25–16	0.03–16	0.125–32	0.03–32	0.03–32	0.02–32
	MIC ₅₀	0.25	2	4	4	1	16	1.25	0.19	0.03
	MIC ₉₀	1	16	16	16	16	32	32	32	8
<i>A. infectoria</i> (16)	GM	0.20	0.95	2.38	3.82	0.66	4.55	6.04	4.81	4.00
	range	0.015–32	0.06–16	0.5–16	0.5–16	0.03–16	0.03–32	0.03–32	0.03–32	0.03–32
	MIC ₅₀	0.25	0.5	2	2	1	8	16	32	4
	MIC ₉₀	0.75	16	16	16	16	32	32	32	32
<i>Alternaria</i> spp. (35)	GM	0.28	1.36	2.95	3.75	0.92	5.94	2.74	1.59	0.73
	range	0.015–32	0.06–16	0.5–16	0.25–16	0.03–16	0.03–32	0.03–32	0.03–32	0.03–32
	MIC ₅₀	0.25	1	2	3	1	16	6	2	0.75
	MIC ₉₀	1	16	16	16	16	32	32	32	32

AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; RCZ, ravuconazole; POS, posaconazole; TRB, terbinafine; CAS, caspofungin; MIC, micafungin; AND, anidulafungin.

itraconazole, voriconazole, ravuconazole and posaconazole. The endpoint for amphotericin B, itraconazole, voriconazole, ravuconazole, posaconazole and terbinafine was the antifungal concentration that produced a complete inhibition of visual growth at 48 h (MIC). For the echinocandins, the endpoint was the antifungal concentration that produced a visible change in the morphology of the hyphae compared with the growth control well (minimum effective concentration) at 48 h.

Analysis of results

Statistical analyses were performed to evaluate the differences between the MIC values. In order to approximate a normal distribution, the MICs were transformed to log₂ values. ANOVA tests were performed to evaluate the differences among the two species analysed to all nine antifungals. A *P* value of <0.01 was considered statistically significant. The analyses were done with the Statistical Package for the Social Sciences (version 18.0; SPSS SL, Madrid, Spain).

Results and discussion

Thirty-five isolates were included in the study. Nineteen were identified as *A. alternata* and 16 as *A. infectoria*. The results of the antifungal susceptibility testing are summarized in Table 1. Statistical analyses showed no significant differences among these two species for all antifungals. However, this could be due to the small sample size. Among all the drugs tested, amphotericin B was the antifungal with the best *in vitro* activity against both species; the geometric mean (GM) of the MICs was 0.36 mg/L for *A. alternata* and 0.20 mg/L for *A. infectoria*. Other studies have shown similar results.^{6,7} Moreover, clinical cases of successful treatment with this drug have been reported.^{8,9} Nevertheless, higher MICs of amphotericin B have also been described in the literature,¹⁰ although none of these studies performed identification to the species level. *A. alternata* showed higher MICs than *A. infectoria* except to ravuconazole (although these differences were low and not statistically significant). While previous studies showed good *in*

vitro activity to voriconazole,⁷ in this collection of clinical isolates the GMs for both species were ≥2 mg/L. Nonetheless, 10 out of 35 isolates showed MICs <2 mg/L. Posaconazole was the most active azole, with an MIC₅₀ of 1 mg/L for both species and a GM for the genus of 0.92 mg/L. It was more active against *A. infectoria* (GM=0.66 mg/L) than against *A. alternata* (GM=1.23 mg/L). Similar results were found in previous studies.⁷ The biggest differences in the azole susceptibility between these species were found in itraconazole. More than half (10 out of 19) of the isolates of *A. alternata* showed high MICs of this drug (GM=1.79 and MIC₅₀=2 mg/L), while only 25% (4 out of 16) of the *A. infectoria* isolates showed high *in vitro* MICs of itraconazole (GM=0.95 mg/L and MIC₅₀=0.5 mg/L). Previous studies showed good activity of itraconazole,^{4,7,10} however, clinical failure has also been described.^{8,9} One of the reasons for these failures could be the presence of resistant isolates, as found in the present and previous studies.¹ Terbinafine generally showed poor activity against both species, with only a small number of strains demonstrating low MICs. The echinocandins are the most recent family of antifungals; thus, there are scarce data available in the literature. The biggest differences in susceptibility between these two species were found in this class of drugs (although these differences were not statistically significant). Thus, all GMs and MIC₅₀s for *A. infectoria* were ≥4 mg/L, while all echinocandins showed good activity against *A. alternata* (GMs and MIC₅₀s <2 mg/L). Anidulafungin was the most active drug in this class and caspofungin was the least active.

An optimal treatment for infections caused by *Alternaria* spp. is not defined. In this study, only the echinocandins showed a different susceptibility pattern among these species, although the spectrum of activity of all antifungals was not uniform. Therefore, susceptibility testing seems to be an important tool in choosing the most active antifungal agent for use against isolates causing such infections.

Funding

This work was supported in part by research projects PI05/32 from the Instituto de Salud Carlos III and by the Spanish Network for Research in Infectious Diseases (REIPI RD06/0008). I. C. has a contract from the Spanish Network for Research in Infectious Diseases (REIPI RD06/0008).

Transparency declarations

In the past 5 years, J. L. R.-T. has received grant support from Astellas Pharma, Gilead Sciences, Merck Sharp and Dohme, Pfizer, Schering-Plough, Soria Melguizo SA, the European Union, the Spanish Agency for International Cooperation, the Spanish Ministry of Culture and Education, the Spanish Health Research Fund, the Instituto de Salud Carlos III, the Ramon Areces Foundation and the Mutua Madrileña Foundation. He has been an advisor/consultant to the Panamerican Health Organization, Gilead Sciences, Merck Sharp and Dohme, Mycognostica, Pfizer and Schering-Plough. He has been paid for talks on behalf of Gilead Sciences, Merck Sharp and Dohme, Pfizer and Schering-Plough. All other authors: none to declare.

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