ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 2011, p. 1794–1797 Vol. 55, No. 4
0066-4804/11/$12.00 doi:10.1128/AAC.01757-10

Frequency of Voriconazole Resistance *In Vitro* among Spanish Clinical Isolates of *Candida* spp. According to Breakpoints Established by the Antifungal Subcommittee of the European Committee on Antimicrobial Susceptibility Testing▽

M. Cuenca-Estrella,* A. Gomez-Lopez, I. Cuesta, O. Zaragoza, E. Mellado, and J. L. Rodriguez-Tudela

Servicio de Micología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Ctra. Majadahonda-Pozuelo Km. 2, 28220 Majadahonda (Madrid), Spain

Received 16 December 2010/Returned for modification 13 January 2011/Accepted 24 January 2011

A total of 4,226 Spanish clinical isolates of *Candida* spp. were analyzed to assess resistance to voriconazole according to breakpoints established by the European Committee for Antimicrobial Susceptibility Testing (where susceptibility [S] to voriconazole corresponds to a MIC of ≤0.12 mg/liter). Resistance was uncommon among *Candida albicans* (5%), *C. parapsilosis* (1.2%), and *C. tropicalis* (11%) isolates. Voriconazole MICs of >0.12 mg/liter were more frequent among *Candida glabrata* and *C. krusei* isolates. A significant percentage of voriconazole-resistant strains came from oropharyngeal infections and exhibited high MICs of other azoles.

The Subcommittee on Antifungal Susceptibility of the European Committee for Antimicrobial Susceptibility Testing (AFST-EUCAST) has determined breakpoints for voriconazole for *Candida* species. The clinical breakpoints have been set for intravenous and oral doses (18). The *in vitro* activity of this azole agent against *Candida* spp. is not uniform. Several studies have reported that the species of *Candida* most frequently involved in human infections, *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Candida glabrata*, and *Candida krusei*, usually exhibit low MICs of voriconazole, although the voriconazole MICs for strains with resistance to fluconazole are proportionally higher than are those for fluconazole-susceptible isolates (3–5, 13).

The EUCAST has developed a standard procedure to set interpretative breakpoints for antimicrobial susceptibility testing (AST). The clinical breakpoints define the organism as susceptible (S), intermediate (I), and resistant (R) to antifungal drugs. The susceptibility and resistance categories are related to high likelihoods of clinical success and clinical failure, respectively. The EUCAST has also defined epidemiological cutoff values (ECOFF values, or ECVs) which are based on the wild-type distributions of MICs for microorganisms. These ECOFF values can help to determine breakpoints when there is limited statistical support for correlation of clinical response with MICs (7, 8, 12).

Wild-type microorganisms are defined by the absence of acquired and mutational mechanisms of resistance to the antifungal. With the distribution of the wild type and its highest MIC having been determined, organisms with acquired or mutational resistance mechanisms can be identified readily as organisms with reduced susceptibility compared with the highest MIC for the wild type. These organisms are called the non-wild-type population. The EUCAST has defined the MIC encompassing the wild-type population as the ECOFF value. The MICs of voriconazole for defining wild-type *Candida* spp. are ≤0.125 mg/liter for *C. albicans*, *C. tropicalis*, and *C. parapsilosis* and ≤1 mg/liter for *C. glabrata* and *C. krusei* (6, 15).

A clinical response of 76% was achieved for infections due to *C. albicans*, *C. tropicalis*, and *C. parapsilosis* when the MICs were lower than or equal to 0.12 mg/liter (9, 10, 14). Wild-type populations of those species were therefore considered to be susceptible to voriconazole, and the EUCAST clinical MIC breakpoints for voriconazole have been set at ≤0.12 mg/liter for defining clinical susceptible isolates and at >0.12 mg/liter for resistant isolates. There is insufficient information on the response to voriconazole treatment in infections caused by *Candida* isolates with higher MICs since pharmacokinetic values are variable and clinical data on species/isolates with MICs in the range of 0.25 to 1.0 mg/liter are scarce. These breakpoints are tentative and will be reviewed after 2 years (18).

Regarding *C. glabrata* and *C. krusei*, the AFST-EUCAST considers that there is insufficient evidence that these species are good targets for therapy with voriconazole, and clinical breakpoints have not been established yet. Clinical studies of systemic candidiasis caused by *C. glabrata* have shown a 21% lower response to voriconazole than the response observed for infections by *C. albicans*, *C. tropicalis*, or *C. parapsilosis* (14, 15).

We describe the occurrence of *in vitro* resistance to voriconazole according to EUCAST breakpoints among clinical isolates of *Candida* spp. collected in a Spanish reference laboratory. The *in vitro* activity of other antifungal agents was also determined for comparative reasons.

(This work was presented in part at the 50th Interscience Conference on Antimicrobial Agents and Chemotherapy, Boston, MA, 2010.)

* 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: 34-91-8223726. Fax: 34-91-5097966. E-mail: mcuenca-estrella@isciii.es.

▽ Published ahead of print on 31 January 2011.

TABLE 1. Voriconazole MIC distribution for each species of *Candida*

Species	Total no. of strains	No. of strains with indicated voriconazole MIC (mg/liter)									
		≤0.015	0.03	0.06	0.12	0.25	0.5	1.0	2.0	4.0	8.0	>8.0
Candida albicans	1,898	1,696	45	32	29	34	19	14	5	2	7	15
Candida parapsilosis	925	792	84	23	14	6	3	1	1	0	0	1
Candida tropicalis	480	229	131	51	16	19	2	5	5	1	3	18
Candida glabrata	682	8	39	141	211	126	66	30	17	26	11	7
Candida krusei	241	3	3	5	25	97	82	20	5	1	0	0

A total of 4,226 *Candida* clinical isolates were analyzed. The strains were recovered from 115 Spanish hospitals over a period of 7 years, from 2003 to 2009. Each isolate came from a different patient. Isolates were identified by morphological and biochemical methods and sequencing of DNA targets if necessary (2). Briefly, for molecular identification purposes, genomic DNA was directly prepared from a single yeast colony. DNA segments comprising the D1/D2 domains of the 26S ribosomal DNA and the internal transcribed spacer 1 (ITS1)/ITS2 regions were amplified and sequenced using universal primers. Further analysis was performed by comparison with the ITS sequences of the type and reference isolates and with those included in the database of the Mycology Department of the Spanish National Centre for Microbiology, a restricted database including more than 6,000 sequenced organisms. Analyses were conducted with InfoQuest FP 4.50 software (Bio-Rad Laboratories, Madrid, Spain).

Species were distributed as follows: 1,898 strains were *C. albicans*, 925 *C. parapsilosis*, 480 *C. tropicalis*, 682 *C. glabrata*, and 241 *C. krusei.* Around 70% of the isolates were isolated from blood cultures and other deep sites, such as tissue samples and internal body fluids, 10% were isolated from oropharyngeal exudates, and the remaining 20% were isolated from vaginal exudates, skin samples, and other specimens.

Susceptibility testing experiments were done strictly in accordance with the reference procedure for testing fermentative yeasts established by the AFST-EUCAST (16). The antifungal agents used were amphotericin B (Sigma-Aldrich Quimica SA, Madrid, Spain) and flucytosine (Sigma-Aldrich), the azoles were fluconazole (Pfizer SA, Madrid, Spain), itraconazole (Janssen SA, Madrid, Spain), posaconazole (Schering-Plough, Kenilworth, NJ), and voriconazole (Pfizer SA), and the echinocandins were anidulafungin (Pfizer SA), micafungin (Astellas Pharma, Inc., Tokyo, Japan), and caspofungin (Merck & Co., Inc., Rahway NJ).

Descriptive and comparative analyses were done. The significance of the differences between MICs was determined by analysis of variance (ANOVA; Bonferroni's *post hoc* test) or nonparametric tests. Differences in proportions were determined by Fisher's exact test or by chi-square analysis. A P value of <0.01 was considered significant.

The number of isolates with voriconazole MICs of >0.12 mg/liter was 649 out of 4,226 (15.3%). The distribution of voriconazole-resistant isolates by species was as follows: 96/1,898 (5%) of *C. albicans* isolates, 12/925 (1.2%) of *C. parapsilosis* isolates, and 53/480 (11%) of *C. tropicalis* isolates were voriconazole resistant. The rates of resistance according the EUCAST clinical breakpoint for *C. glabrata* and *C. krusei* were 41% (283/682) and 85% (205/241), respectively.

Table 1 displays the distribution of MICs of voriconazole for each species of *Candida*. Table 2 shows voriconazole MICs stratified by fluconazole susceptibility category (S/I/R) according to EUCAST fluconazole breakpoints (17). Strains exhibiting *in vitro* resistance to voriconazole showed high MICs of fluconazole and other azole agents ($P < 0.01$; ANOVA). Table 3 shows percentages (by species and by clinical specimen) of clinical strains with MICs above 0.12 mg/liter, the clinical breakpoint and ECOFF value for *C. albicans*, *C. tropicalis*, and *C. parapsilosis*. In addition, the table includes percentages of strains with MICs above 1 mg/liter, the ECOFF value determined by the EUCAST for *C. glabrata* and *C. krusei*. The number of isolates exhibiting voriconazole MICs of >1 mg/liter was 125 (2.9%), of which 61 (49%) were *C. glabrata*.

By clinical origin, *C. albicans* strains with *in vitro* resistance

TABLE 2. Voriconazole MICs according to EUCAST fluconazole breakpoints[a]

Species	Voriconazole MIC (mg/liter) for indicated fluconazole susceptibility category[b]								
	S (n = 3,247)			I (n = 261)			R (n = 718)		
	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range
Candida albicans	0.015	0.015	0.015–1.0	0.03	1.0	0.015–1.0	0.25	>8.0	0.015–>8.0
Candida parapsilosis	0.015	0.03	0.015–0.12	0.12	0.25	0.015–0.25	0.25	2.0	0.03–>8.0
Candida tropicalis	0.015	0.06	0.015–0.50	0.25	2.0	0.015–2.0	2.0	>8.0	0.015–>8.0
Candida glabrata	0.06	0.25	0.015–0.50	0.12	0.25	0.03–0.50	0.25	4.0	0.015–>8.0
Candida krusei[c]							0.25	1.0	0.015–4.0
Total	0.015	0.03	0.015–0.50	0.12	0.25	0.015–2.0	0.50	4.0	0.015–>8.0

[a] See reference 17.

[b] S, fluconazole-susceptible strains (MIC ≤ 2); I, fluconazole-intermediate strains (MIC = 4); R, fluconazole-resistant strains (MIC > 8); MIC_{50}, concentration causing inhibition of 50% of isolates; MIC_{90}, concentration causing inhibition of 90% of isolates.

[c] *C. krusei* is intrinsically resistant to fluconazole.

TABLE 3. Rates of voriconazole resistance *in vitro* among species and clinical specimens

Species and source of clinical specimen	No. of strains	No. (%) of strains with indicated MIC (mg/liter)	
		>0.12[c]	>1[d]
Candida albicans			
Deep sites[a]	1,116	27 (2.4)	15 (1.3)
Oropharynx	300	61 (20.3)	13 (4.3)
Other[b]	482	8 (1.6)	1 (0.2)
Total	1,898	96 (5)	29 (1.5)
Candida parapsilosis			
Deep sites[a]	726	8 (1.1)	1 (0.1)
Oropharynx	13	1 (7.7)	0 (0)
Other[b]	186	3 (1.6)	1 (0.5)
Total	925	12 (1.2)	2 (0.2)
Candida tropicalis			
Deep sites[a]	376	39 (10.4)	21 (5.6)
Oropharynx	20	2 (10)	1 (5)
Other[b]	84	12 (14.3)	5 (5.9)
Total	480	53 (11)	27 (5.6)
Candida glabrata			
Deep sites[a]	431	172 (39.9)	37 (8.6)
Oropharynx	36	20 (55.5)	7 (19.4)
Other[b]	215	91 (42.3)	17 (7.9)
Total	682	283 (41.2)	61 (8.9)
Candida krusei			
Deep sites[a]	157	134 (85.3)	3 (1.9)
Oropharynx	33	28 (84.8)	2 (6.1)
Other[b]	51	43 (84.3)	1 (1.9)
Total	241	205 (85)	6 (2.5)

[a] Deep sites included blood cultures, tissue biopsy specimens, and internal body fluids.
[b] Other sources included vaginal exudates and skin, hair, and nail samples.
[c] The voriconazole clinical breakpoint and ECOFF value defined by EUCAST for *C. albicans*, *C. parapsilosis*, and *C. tropicalis*.
[d] The voriconazole ECOFF value defined by EUCAST for *C. glabrata* and *C. krusei*.

to voriconazole were significantly associated with oropharyngeal infections, as 61 resistant *C. albicans* organisms were isolated from patients suffering from that infection (61/96 [63%] [$P < 0.01$]; odds ratio [OR], 13.8 to 21.8 [chi-square analysis]). A total of 300 *C. albicans* clinical strains were collected from oropharyngeal samples, and 20% of these strains were found to be resistant (61/300 isolates). Voriconazole-resistant isolates of other *Candida* species were observed irrespective of the sample analyzed. Resistance to voriconazole in *C. glabrata* was not associated with vaginal samples either, since comparable resistance rates were observed for blood cultures, deep-site samples, oropharyngeal exudates, and vaginal exudates. It should be noted that the emergence of resistance *in vitro* was not detected when analysis was done according to year of isolation.

According to the EUCAST breakpoint, *in vitro* resistance to voriconazole was infrequent among Spanish clinical isolates of *C. albicans* and *C. parapsilosis*. Resistance to this azole was somehow more common in *C. tropicalis* (11%). It should be noted that the *C. tropicalis* resistance rate may be biased, as reference laboratories receive uncommon species, microorganisms that are often difficult to identify, and resistant isolates for AST.

The determination of clinical breakpoints by the EUCAST has been based on dosage, pharmacokinetic, and pharmacodynamic data for voriconazole (18). The intravenous dose of this azole for adults is 4 mg/kg of body weight twice daily, and the oral dose for patients weighing >40 kg is 200 mg twice daily. Loading doses are recommended as well, 12 mg/kg/day intravenously or 400 mg twice a day for oral administration on the first day of therapy. The pharmacokinetic values for voriconazole are nonlinear and variable. Concentrations in plasma differ >100-fold among subjects, depending on the genotype of the hepatic cytochrome P450, interacting medication, and other factors. The index representing the area under the concentration-time curve for the free, unbound fraction of a drug divided by the MIC (*f*AUC/MIC) is the parameter best related to outcome. Animal models and Monte Carlo simulations have shown that a target *f*AUC/MIC of 24 would inhibit 99% of isolates with voriconazole MICs of ≤0.25 mg/liter (1, 11).

Taking into account these results, the EUCAST established the voriconazole breakpoint as stated above (18). That breakpoint is not applicable for *C. glabrata* and *C. krusei* isolates, since there is insufficient evidence that those species are good targets for therapy with voriconazole. In the case of *C. glabrata*, MIC analysis did not find the explanation for the lower clinical response, and there were only nine cases of *C. krusei* available for analysis. Consequently, clinical breakpoints for *C. glabrata* and *C. krusei* have not been determined, and more data should become available for setting them.

An ECOFF value for these species has been established at ≤1 mg/liter by following the wild-type distribution. According to that value, the rates of non-wild-type populations among Spanish isolates are low for *C. glabrata* and *C. krusei*, amounting to 9% and 2.5%, respectively. However, rates of resistance *in vitro* are significantly higher if the clinical breakpoint value (≤0.12 mg/liter) is used to classify these species, as 41% of *C. glabrata* strains and 85% of *C. krusei* strains exhibited MICs of >0.12 mg/liter. It should be prominently indicated that results achieved with passive epidemiological surveillance performed by reference centers can be biased, as those laboratories receive uncommon species and microorganisms that are difficult to identify.

In conclusion, *in vitro* resistance to voriconazole is uncommon among Spanish isolates of *C. albicans*, *C. parapsilosis*, and *C. tropicalis*. Most of the isolates exhibiting voriconazole resistance were *C. albicans* isolates from oropharyngeal infections and with cross-resistance to other azole agents. Higher MICs of voriconazole were more frequently observed among isolates of *C. glabrata* and *C. krusei*, and these species could be a bad target for therapy with voriconazole.

This study was cofinanced by a nonrestrictive grant from Pfizer SA. The study was supported by Ministerio de Ciencia e Innovación, Instituto de Salud Carlos III (cofinanced by the European Development Regional Fund [ERDF] "A way to achieve Europe"), and by the Spanish Network for the Research in Infectious Diseases (REIPI RD06/0008). I.C. has a research contract with the REIPI.

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 Pa-

namerican Health Organization, Astellas Pharma, 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. In the past 5 years, M.C.-E. has received grant support from Astellas Pharma, bioMerieux, Gilead Sciences, Merck Sharp and Dohme, Pfizer, Schering Plough, Soria Melguizo SA, the European Union, the ALBAN program, 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, Astellas Pharma, Gilead Sciences, Merck Sharp and Dohme, Pfizer, and Schering Plough. He has been paid for talks on behalf of Gilead Sciences, Merck Sharp and Dohme, Pfizer, Astellas Pharma, and Schering Plough.

REFERENCES

1. **Andes, D., A. Pascual, and O. Marchetti.** 2009. Antifungal therapeutic drug monitoring: established and emerging indications. Antimicrob. Agents Chemother. **53:**24–34.
2. **Cendejas-Bueno, E., A. Gomez-Lopez, E. Mellado, J. L. Rodriguez-Tudela, and M. Cuenca-Estrella.** 2010. Identification of pathogenic rare yeast species in clinical samples: comparison between phenotypical and molecular methods. J. Clin. Microbiol. **48:**1895–1899.
3. **Cuenca-Estrella, M., et al.** 2006. Head-to-head comparison of the activities of currently available antifungal agents against 3,378 Spanish clinical isolates of yeasts and filamentous fungi. Antimicrob. Agents Chemother. **50:**917–921.
4. **Cuenca-Estrella, M., et al.** 2009. Analysis of the activity profile in vitro of micafungin against spanish clinical isolates of common and emerging species of yeasts and molds. Antimicrob. Agents Chemother. **53:**2192–2195.
5. **Cuenca-Estrella, M., et al.** 2005. In vitro susceptibilities of bloodstream isolates of Candida species to six antifungal agents: results from a population-based active surveillance programme, Barcelona, Spain, 2002–2003. J. Antimicrob. Chemother. **55:**194–199.
6. **Cuenca-Estrella, M., and J. L. Rodriguez-Tudela.** 2010. The current role of the reference procedures by CLSI and EUCAST in the detection of resistance to antifungal agents in vitro. Expert Rev. Anti Infect. Ther. **8:**267–276.
7. **Kahlmeter, G., et al.** 2006. European Committee on Antimicrobial Susceptibility Testing (EUCAST) Technical Notes on antimicrobial susceptibility testing. Clin. Microbiol. Infect. **12:**501–503.
8. **Kahlmeter, G., et al.** 2003. European harmonization of MIC breakpoints for antimicrobial susceptibility testing of bacteria. J. Antimicrob. Chemother. **52:**145–148.
9. **Kullberg, B. J., et al.** 2005. Voriconazole versus a regimen of amphotericin B followed by fluconazole for candidaemia in non-neutropenic patients: a randomised non-inferiority trial. Lancet **366:**1435–1442.
10. **Ostrosky-Zeichner, L., A. M. Oude Lashof, B. J. Kullberg, and J. H. Rex.** 2003. Voriconazole salvage treatment of invasive candidiasis. Eur. J. Clin. Microbiol. Infect. Dis. **22:**651–655.
11. **Pascual, A., et al.** 2008. Voriconazole therapeutic drug monitoring in patients with invasive mycoses improves efficacy and safety outcomes. Clin. Infect. Dis. **46:**201–211.
12. **Pfaller, M. A., D. Andes, D. J. Diekema, A. Espinel-Ingroff, and D. Sheehan.** 2010. Wild-type MIC distributions, epidemiological cutoff values and species-specific clinical breakpoints for fluconazole and Candida: time for harmonization of CLSI and EUCAST broth microdilution methods. Drug Resist. Updat. **13:**180–195.
13. **Pfaller, M. A., and D. J. Diekema.** 2007. Epidemiology of invasive candidiasis: a persistent public health problem. Clin. Microbiol. Rev. **20:**133–163.
14. **Pfaller, M. A., et al.** 2006. Correlation of MIC with outcome for Candida species tested against voriconazole: analysis and proposal for interpretive breakpoints. J. Clin. Microbiol. **44:**819–826.
15. **Rodriguez-Tudela, J. L., M. C. Arendrup, M. Cuenca-Estrella, J. P. Donnelly, and C. Lass-Florl.** 2010. EUCAST breakpoints for antifungals. Drug News Perspect. **23:**93–97.
16. **Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID European Committee for Antimicrobial Susceptibility Testing (EUCAST).** 2008. EUCAST Definitive Document EDef 7.1: method for the determination of broth dilution MICs of antifungal agents for fermentative yeasts. Clin. Microbiol. Infect. **14:**398–405.
17. **Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID European Committee for Antimicrobial Susceptibility Testing (EUCAST).** 2008. EUCAST technical note on fluconazole. Clin. Microbiol. Infect. **14:** 193–195.
18. **Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID European Committee for Antimicrobial Susceptibility Testing (EUCAST).** 2008. EUCAST Technical Note on voriconazole. Clin. Microbiol. Infect. **14:**985–987.