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Colorimetric method for susceptibility testing of voriconazole and other triazoles against *Candida* species

Kolorimetrische Methode zur Empfindlichkeitsprüfung von Voriconazol und anderen Triazolen für *Candida*-Arten

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Key words. *Candida*, susceptibility testing, voriconazole, triazoles, colorimetric assay, Alamar Blue.
Schlüsselwörter. *Candida*, Empfindlichjeitsprüfung, Voriconazol, Triazole, kolorimetrische Methode, Almarblau.

Summary. A microdilution assay using Alamar Blue, a colorimetric indicator, was compared with the NCCLS macrodilution broth assay for voriconazole, fluconazole, and itraconazole against *Candida albicans*, *Candida glabrata*, and *Candida krusei*. Concordance (± 2 dilutions) between the two methods was highest for voriconazole (98.3%), and for fluconazole and itraconazole it was 94.3 and 95.4%, respectively. Twenty-six of 32 (81.2%) discordant readings (≥ 3 dilutions different) were noted in *C. glabrata* isolates, and all but two isolates showing discordance had higher minimum inhibitory concentration readings with the colorimetric method.

Zusammenfassung. Ein Mikrodilutionstest unter Verwendung des Farbindikators Alamarblau wurde mit dem NCCLS-Makrodilutionstest zur Empfindlichkeitsprüfung von *Candida albicans*, *C. glabrata* und *C. krusei* für Voriconazol, Fluconazol und Itraconazol verglichen. Die Übereinstimmung (± 2 Verdünnungsstufen) war am besten bei Voriconazol (98.3%), gefolgt von Fluconazol (94.3%) und Itraconazol (95.4%). Diskordante Ablesungen (≥ 3 Verdünnungsstufen) traten 26 mal (81.2%) bei *C. glabrata*-Isolaten auf, aber außer zwei zeigten alle diskordanten Isolate höhere MHK-Werte mit der kolorimetrischen Methode.

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Introduction

Voriconazole is a new triazole agent that appears to have a broader spectrum of antifungal activity than the parent compound, fluconazole [1–6]. Prior studies that have assessed the *in vitro* activity of voriconazole against *Candida* species have utilized the National Committee for Clinical Laboratory Standards (NCCLS) recommended microdilution or macrodilution methods. However, the visual turbidity endpoints of these assays are frequently difficult to define for azole agents. Several species, most notably *Candida albicans*, exhibit a trailing phenomenon rather than a sharp end-point [7, 8].

Alamar Blue (AccuMed, Westlake, OH, USA) is an oxidation–reduction colorimetric indicator that, in the presence of actively growing organisms, changes colour from a deep blue to a bright pink. We and others have previously shown the feasibility of using this system for antifungal susceptibility testing [9–14]. Voriconazole has not previously been tested with this method.

In this study, we compared the results obtained using a colorimetric microdilution method with the NCCLS macrodilution method for isolates of *C. albicans*, *Candida glabrata* and *Candida krusei* against voriconazole, as well as fluconazole and itraconazole.

Methods

Antifungal drugs

The stock solution of voriconazole (Pfizer, Inc., Groton, CT, USA) was made by dissolving

the powder in dimethyl sulfoxide (DMSO). Fluconazole (Pfizer, Inc.) was dissolved in sterile distilled water. Itraconazole (Janssen Research Foundation, Beerse, Belgium) was solubilized in DMSO. All stock solutions were stored at −70 °C and then thawed and diluted in RPMI-1640 (Sigma, St. Louis, MO, USA) on the day that the test was performed.

Fungal isolates

Isolates of *C. albicans*, *C. glabrata* and *C. krusei* were obtained between 1981 and 1997 from our patient populations, including those who had neutropenia [15, 16] or HIV infection [17] and those who were in an intensive care unit or a nursing home [18]. The isolates from the 1980s had been stored at room temperature in distilled water in air-tight containers, and the isolates from 1990 onward were stored at −70 °C in Sabouraud glucose broth with glycerol. Each isolate was retrieved from storage and plated twice in succession on Sabouraud glucose agar prior to testing. Two control strains, *C. glabrata* ATCC 90030 and *Candida parapsilosis* ATCC 90018, were tested on each day that the minimum inhibitory concentration (MIC) levels were determined.

Susceptibility testing

The NCCLS broth macrodilution method was performed as outlined in the M27-A document [19]. Briefly, RPMI 1640 medium with morpholinepropanesulfonic acid (MOPS) buffer (Sigma), was used at a final pH of 7.0. The final inoculum was 0.5×10^3 to 2.5×10^3 yeast cells ml^{-1}; the total volume was 1 ml. Tubes were incubated at 35 °C for 48 h. The MIC was the drug concentration at which visual turbidity was less than or equal to that of the comparison tube that consisted of a 1 : 5 dilution of the growth control (80% growth suppression). Concentrations of fluconazole ranged from 0.06 to 64 μg ml^{-1}; itraconazole and voriconazole ranged from 0.007 to 4 μg ml^{-1}.

The Alamar Blue modified NCCLS microdilution method was performed as described previously [12]. The reagents, medium preparation, inoculum and drug concentrations were the same as noted above for the macrodilution method with two exceptions: Alamar Blue (1 μl of 100 × reagent) was added to each 1 ml of RPMI 1640-MOPS medium prior to inoculation of the yeast, and U-bottom, 96-well, microtitre plates were used for the assay. To each well was added 180 μl of RPMI 1640–MOPS with Alamar Blue containing 0.5×10^3 to 2.5×10^3 yeast cells and 20 μl of the appropriate concentration of drug. The plates were incubated at 35 °C for 48 h. The MIC was determined as the lowest drug concentration that maintained a blue or a blue–pink hue. Wells with growth were pink–red in colour.

Results

A total of 137 isolates of *C. albicans*, 124 isolates of *C. glabrata*, and 20 isolates of *C. krusei* were studied by both methods. Comparing the two methods, MIC_{50} and MIC_{90} results differed by ≤ 1 dilution for the three triazoles for each of the three *Candida* species (Table 1). Major discordance (≥ 3 dilutions different) between the two methods occurred on only 32 determinations (Table 2). The discordance was most marked for *C. glabrata* (26 of 32 discordant readings) and with MIC determinations for itraconazole and fluconazole. Only three isolates (all *C. albicans*) showed major discor-

Table 1. Comparison of triazole susceptibilities obtained with NCCLS macrodilution method versus the Alamar Blue microdilution method

Organism (no.)	Minimum inhibitory concentration (μg ml^{-1})								
	Voriconazole			Itraconazole			Fluconazole		
	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}
C. albicans (137)									
macro	0.007–2	0.01	0.03	0.01–>4	0.06	0.12	0.06–>64	0.25	1
Alamar	0.007–2	0.01	0.06	0.01–>4	0.06	0.12	0.12–32	0.5	1
C. glabrata (124)									
macro	0.06–>4	1	2	0.06–>4	1	4	0.5–>64	8	32
Alamar	0.06–>4	1	2	0.25–>4	1	4	2–>64	16	32
C. krusei (20)									
macro	0.25–2	0.5	2	0.12–>4	2	4	32–>64	64	>64
Alamar	0.25–2	1	2	0.25–>4	2	4	32–>64	64	>64

Table 2. Concordance of Alamar Blue colorimetric microdilution method with NCCLS macrodilution method

Organism (no.)	Voriconazole		Itraconazole		Fluconazole	
	±2 dil	±3 dil	±2 dil	±3 dil	±2 dil	±3 dil
C. albicans (137)	126 (92)	134 (98)	123 (90)	136 (99)	122 (89)	135 (99)
C. glabrata (124)	116 (94)	124 (100)	100 (81)	112 (90)	99 (80)	110 (89)
C. krusei (20)	17 (85)	20 (100)	19 (95)	20 (100)	20 (100)	20 (100)

Results expressed as number (%) of isolates in each dilution range in which MIC values obtained by the colorimetric method were concordant with those obtained by the NCCLS method.

dance for voriconazole. For 30 of the 32 major discordant readings, the colorimetric reading was higher than the NCCLS macrodilution reading. For all three triazoles, the colorimetric change was not completed by 24 h for many isolates, especially *C. glabrata* isolates, which tended to grow more slowly. By 48 h, the colour changes were clear-cut and able to be read reproducibly. Some, but not all, isolates that demonstrated trailing and were difficult to read by the 80% visual turbidity endpoint also showed gradations of mauve-to-purple, rather than clear-cut red–blue endpoints, by the colorimetric assay.

Discussion

In this study, we found that a colorimetric assay utilizing the Alamar Blue reagent was comparable with the NCCLS macrodilution broth method for voriconazole, as well as the other triazoles, fluconazole and itraconazole. Major discrepancies between the two methods were uncommon and were found most often for *C. glabrata*. With both methods, the voriconazole endpoints were the most clear-cut of the three triazoles. This could reflect the ability of this new triazole to more effectively inhibit yeasts when compared with the older triazoles.

We verified by the colorimetric method the results of an earlier study using some of these same isolates [4] that voriconazole had lower MIC_{50} values than fluconazole for all three species of *Candida*, and had lower MIC_{50} values than itraconazole for both *C. albicans* and *C. krusei*, but not for *C. glabrata*. These data are similar to those reported previously by others [1–3, 5].

The NCCLS method has gained wide acceptance and has standardized the approach to yeast susceptibility testing. However, the visual endpoints for both micro and macro methods may be difficult to read. The colorimetric method utilizing Alamar Blue offers a different method for endpoint determination. The colorimetric endpoints were easier to read than the turbidity endpoints of the NCCLS method. However, there were problematic isolates of *C. albicans* that showed trailing by the NCCLS method and that also demonstrated 'colorimetric trailing', manifested by mauve hues rather than discrete red–blue endpoints, by the Alamar Blue assay.

Recently, Rex *et al.* in an attempt to correlate *in vitro* susceptibility testing with *in vivo* efficacy in an animal model, noted that 24-h MIC readings may be more appropriate than 48-h readings for *Candida* species [8]. Similarly, Revankar *et al.* identified several strains that exhibited trailing and for which 24-h readings matched better with clinical outcome than 48-h readings [7]. However, we and others [14] have found that the colorimetric readings were not fully developed at 24 h, especially for the more slowly growing *C. glabrata* isolates. Thus, for 48-h readings, the Alamar Blue colorimetric assay appears to be a useful addition to yeast susceptibility testing, but 24-h endpoints were not enhanced with this method. If further correlations of *in vivo* response to triazoles show that 24-h MIC readings correlate better with outcome, then the colorimetric method will not enhance *in vitro* susceptibility testing.

Acknowledgements

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