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Reliability of the Vitek 2 Yeast Susceptibility Test for Detection of In Vitro Resistance to Fluconazole and Voriconazole in Clinical Isolates of *Candida albicans* and *Candida glabrata*▽†

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The Vitek 2 yeast susceptibility test was evaluated by testing 122 *Candida* isolates against fluconazole and voriconazole. Excellent categorical agreement with the CLSI broth microdilution method was observed (97.5% for both the azoles). Moreover, the Vitek 2 system was able to identify all but 2 of 59 investigated fluconazole-resistant organisms.

Whereas fluconazole and, more recently, voriconazole have been widely used for the prophylaxis and therapy of candidiasis (16, 21, 28), detection of *Candida* isolates resistant in vitro to these agents has become clinically significant, particularly in patient populations on prophylactic regimens or in settings of frequent isolation of *Candida* species (e.g., *Candida glabrata*) with unpredictable patterns of drug susceptibility (17). The Clinical and Laboratory Standards Institute (CLSI) (formerly the National Committee for Clinical Laboratory Standards) broth microdilution (BMD) methods for in vitro susceptibility testing of *Candida* species against fluconazole and voriconazole have been available since 1997 and 2002, respectively. These methods remain the standard techniques for susceptibility testing in many clinical laboratories, but they are labor intensive because of in-house plate preparation, which requires tedious dilution procedures. For this reason, simpler and more commercially available antifungal susceptibility testing systems, such as the Sensititre YeastOne system (Trek Diagnostic Systems, Cleveland, OH) and the Etest (AB Biodisk, Solna, Sweden), have been developed and approved by the U.S. Food and Drug Administration for routine use in the clinical laboratory.

In an effort to automate antifungal susceptibility testing, bioMérieux, Inc. (Hazelwood, MO) developed a fully automated antifungal susceptibility testing system (Vitek 2 yeast susceptibility test). Two large multicenter evaluations of the Vitek 2 conducted by Pfaller et al. (18, 19) showed that this system, which relies upon a spectrophotometric approach, provided a highly reproducible, rapid, and accurate means of determining for *Candida* species the MICs not only of fluconazole and voriconazole but also of amphotericin B and flucytosine. In addition, the system was capable of identifying resistance to fluconazole (18) and to voriconazole and flucytosine (19) among various *Candida* species.

The purpose of the present study was to further evaluate the accuracy of the Vitek 2 yeast susceptibility test for detection of antifungal resistance to fluconazole and voriconazole. This evaluation compared the Vitek 2 results to those obtained by the CLSI BMD method and tested *Candida* species with well-characterized resistance mechanisms.

We tested 122 clinical isolates, including 36 *Candida albicans* isolates and 86 *C. glabrata* isolates, that were collected at the University Hospitals of Rome (Italy) and Lausanne (Switzerland) (see Table S1 in the supplemental material). Almost all of *C. albicans* isolates were obtained from oropharyngeal specimens, whereas *C. glabrata* isolates were recovered from different clinical specimens (e.g., blood, urine, vaginal, and sputum). These isolates were selected to represent broad ranges of susceptibilities in vitro, including organisms resistant to fluconazole and/or voriconazole and with documented resistance mechanisms (11, 27, 29). Of note, among the fluconazole-resistant *C. glabrata* isolates, 35 isolates came from clinical sources other than oral ones, including blood (8 isolates), urine (12 isolates), vagina (8 isolates), skin (4 isolates), and bronchoalveolar lavage fluid (3 isolates). Each isolate was from unique infectious episodes from individual patients, with the exception of matched isolates (susceptible, susceptible dose dependent [SDD], and resistant) that were sequentially obtained from the same patients. Isolates were retrieved from frozen (−70°C) storage for testing. Before the tests were performed, each isolate was subcultured onto Sabouraud dextrose agar to ensure its purity and viability and reidentified by standard methods (12). Quality control strains (*Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258) were incorporated in each set of experiments, as recommended by CLSI (4, 7). Antifungal powders (Pfizer, New York, NY) were used to prepare microdilution panels containing serial twofold dilutions of fluconazole (concentration range, 0.125 to 128 μg/ml) and voriconazole (concentration range, 0.03 to 16 μg/ml). Reference BMD testing was performed as outlined in the CLSI document M27-A3 (7), with a final inoculum concentration of 1.5 (±1.0) × 10^3 cells/ml and RPMI 1640 medium

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buffered to pH 7.0 with morpholinepropanesulfonic acid. At 48 h, the lowest concentration of both the azoles that produced a prominent decrease in turbidity (~50% reduction in growth) compared with that of the drug-free growth control was used as the MIC endpoint (7). Susceptibility testing with the Vitek 2 system was performed according to the manufacturer's instructions. From each organism, a standardized 2.0 McFarland inoculum suspension was placed in a Vitek 2 cassette along with a sterile polystyrene test tube and a Vitek 2 card containing serial twofold dilutions of fluconazole (range, 1 to 64 μg/ml) and voriconazole (range, 0.125 to 8 μg/ml). After the loaded cassettes were placed in the Vitek 2 instrument, the cards were filled with the appropriately diluted yeast suspensions, incubated (for a maximum of 24 h), and read automatically. The MIC results (expressed in μg/ml) obtained with the Vitek 2 system were compared with those of the reference BMD. As described in previous works (18, 19), high off-scale MIC results were converted to the next-highest concentration and low off-scale MIC results were left unchanged. The results were considered to be in essential agreement (EA) when the Vitek 2 result was within 2 dilutions (two wells) of the reference value. The percent EA was calculated by using the number of test results in EA as the numerator and the total number of organisms tested as the denominator. The CLSI interpretive breakpoints for fluconazole (susceptible, ≤8 μg/ml; SDD, 16 to 32 μg/ml; resistant, ≥64 μg/ml) and for voriconazole (susceptible, ≤1 μg/ml; SDD, 2 μg/ml; resistant, ≥4 μg/ml) were used to obtain the percent categorical agreement (CA) between the Vitek 2 and reference MICs, which was calculated by dividing the number of tests with no category discrepancy by the number of organisms tested. Very major errors were identified when the reference MIC categorized an isolate as resistant but the Vitek 2 MIC categorized it as susceptible (falsely susceptible), and these were calculated by using the number of resistant isolates as the denominator. Major errors were identified when the reference method categorized the isolate as susceptible but the Vitek 2 method categorized it as resistant, and these were calculated by using the number of susceptible isolates as the denominator. Minor errors were determined when the result of one of the test methods was either susceptible or resistant and that of the other was SDD. The percent minor errors were calculated by using the total number of organisms tested as the denominator. In cases of discrepant results, testing of both methods (the Vitek 2 system and reference BMD) were repeated and the results for the second runs were accepted as the final results.

Expression of the *CDR1*, *CDR2*, *SNQ2*, and *ERG11* genes from *C. glabrata* isolates and of the *CDR1*, *CDR2*, *MDR1*, and *ERG11* genes from *C. albicans* isolates was quantitatively assessed with real-time reverse transcription-PCR in an i-Cycler iQ system (Bio-Rad Laboratories, Hercules, CA), using primers, probes, and thermal conditions described previously (2, 27, 29). For relative quantification of the target genes, each set of primer pairs and the TaqMan probe were used in combination with the primers and probe specific for the *ACT1* (29) or *TEF3* (2) gene in separate reactions. For each isolate, gene expression increases were determined from the mean normalized expression relative to the mean normalized expression of the parental susceptible isolate for related isolates (*C. albicans* and *C. glabrata*) and of the susceptible DSY562 isolate for unrelated isolates (*C. glabrata*) (27). A twofold increase in the expression level of each gene was arbitrarily considered significant. To detect mutations within the genes encoding the lanosterol l4α-demethylase (*ERG11* from *C. albicans* and *ERG11* from *C. glabrata*), DNAs from the respective isolates were amplified by PCR and sequencing according to procedures described previously (24, 27).

The in vitro susceptibilities to fluconazole and voriconazole of 122 clinical isolates of *Candida* species (36 *C. albicans* isolates and 86 *C. glabrata* isolates) were determined by the Vitek 2 system and the BMD reference method read at 24 and 48 h (see Table S1 in the supplemental material). However, only the 48-h BMD result was used as a reference, in accordance with the CLSI procedure that requires holding voriconazole for 48 h prior to determining the MIC, while fluconazole may be read at either 24 or 48 h (7), although several studies (9, 14, 18) have shown the 24-h BMD results to be the most appropriate comparator for Vitek 2 or other systems (e.g., Etest) for fluconazole susceptibility testing, especially with those *Candida* species, such as *C. glabrata*, exhibiting trailing growth (3). Furthermore, a good correlation between 24-h and 48-h fluconazole BMD MICs was documented very recently (15, 20). Another study comparing Etest and Sensititre against the BMD reference method also showed that both the systems provided better agreement at 24 h than at 48 h for *C. glabrata*, although it remained disappointingly low (<80% for fluconazole), suggesting the need for further evaluation of activity of fluconazole (and other azole antifungals) against this *Candida* species (1). For this reason, in the present study we assessed the reliability of the Vitek 2 system by testing a collection of *Candida* isolates (susceptible and resistant in vitro to fluconazole and/or voriconazole), the majority of which (>70%) belonged to *C. glabrata*. Of these isolates, approximately 58% (50 of 86) and 37% (32 of 86) exhibited MICs to fluconazole and voriconazole of ≥64 μg/ml and ≥4 μg/ml, respectively, based on the BMD testing method (see Table S1 in the supplemental material).

The mean time required for a results output in the Vitek 2 system was 14.88 h, with a range from 13 to 19 h. Only two isolates, both azole-resistant petite mutants of *C. glabrata*, failed to grow in the Vitek 2 system and were then excluded from the analysis. In spite of the slower growth of petite mutants than of their parent isolates (6), both the isolates grew well in the BMD wells to be read after only 24 h of incubation. It is plausible that petite mutant isolates show severe growth retardation in specific growth media owing to their respiratory deficiency (6).

Overall, the EA between the Vitek 2 and BMD MIC data ranged from 97.5% (for fluconazole) to 98.3% (for voriconazole). When discrepancies between the Vitek 2 system and the BMD results were carefully examined, we noted that the MICs obtained by the Vitek 2 system were higher than those generated by the BMD method in only five cases (three with fluconazole and two with voriconazole). For both the azoles, the overall CA was 97.5% when the Vitek 2 results were compared to the BMD MICs. Categorical errors included 5% (6 of 120) minor errors and no very major or major errors.

Table 1 summarizes the percent essential and categorical agreements for the comparison between the methods according to the *Candida* species. The lowest EA rates were detected

TABLE 1. Agreement between results obtained by the Vitek 2 yeast susceptibility test and CLSI BMD method, with data classified by *Candida* species[a]

Species (n[b])	Antifungal agent	Test system(s)	% EA	No. (%) of isolates with result			% CA	% Error		
				S	SDD	R		VME	ME	Minor
C. albicans (36)	Fluconazole	Vitek 2		17 (47.2)	8 (22.2)	11 (30.6)				
	Fluconazole	BMD	91.6	17 (47.2)	8 (22.2)	11 (30.6)	100.0	0.0	0.0	0.0
	Voriconazole	Vitek 2		27 (75)	4 (11.1)	5 (13.9)				
	Voriconazole	BMD	94.4	27 (75)	3 (8.3)	6 (16.7)	97.2	0.0	0.0	2.8
C. glabrata (84)[c]	Fluconazole	Vitek 2		23 (27.4)	15 (17.8)	46 (54.8)				
	Fluconazole	BMD	100	24 (28.6)	12 (14.3)	48 (57.1)	96.4	0.0	0.0	3.6
	Voriconazole	Vitek 2		42 (50.0)	13 (15.5)	29 (34.5)				
	Voriconazole	BMD	100	41 (48.8)	13 (15.5)	30 (35.7)	97.6	0.0	0.0	2.4

[a] Abbreviations: EA, essential agreement; S, susceptible; SDD, susceptible, dose dependent; R, resistant; CA, categorical agreement; VME, very major error; ME, major error; minor, minor error.
[b] n, no. of isolates tested.
[c] Of all isolates tested (n = 86), two isolates had MICs not determinable with Vitek 2.

for *C. albicans* tested against fluconazole (three discrepant results). While 100% CA was observed with *C. albicans* for fluconazole, slightly lower CA rates were seen with *C. albicans* for voriconazole (97.2%) and with *C. glabrata* for fluconazole and voriconazole (96.4 and 97.6%, respectively). For *C. albicans*, a minor error regarded only one isolate detected as SDD by Vitek 2 (MIC of 2 μg/ml) and resistant by BMD (MIC of 4 μg/ml). Five minor errors occurred with different *C. glabrata* isolates and were the result of isolates being detected as susceptible by BMD and SDD by Vitek 2 (one case with fluconazole), as susceptible by Vitek 2 and SDD by BMD (one case with voriconazole), and as SDD by Vitek 2 and resistant by BMD (three cases, two with fluconazole and one with voriconazole). As determined with the Vitek 2 system, two of the three resistant isolates had an MIC to fluconazole of 32 μg/ml (versus a BMD MIC of 64 μg/ml) and the remaining one had an MIC to voriconazole of 2 μg/ml (versus a BMD MIC of 4 μg/ml). Importantly, all but three *C. glabrata* isolates exhibiting resistance to fluconazole and/or voriconazole were classified as resistant with the Vitek 2 system.

Our findings were corroborated by the results of molecular analyses performed on the 61 fluconazole-resistant isolates (11 *C. albicans* isolates and 50 *C. glabrata* isolates) included in the present study. In addition to the enhanced drug efflux due to the transcriptional activation of multidrug transporters belonging to the families of ATP-binding cassette (ABC) transporters (encoded by *CDR* genes) and major facilitators (encoded by *MDR* genes), azole resistance in *C. albicans* can be the result of an alteration of the azole target enzyme lanosterol l4α-demethylase via either overexpression or mutation(s) in *ERG11*. In contrast, it is known that azole resistance in *C. glabrata* is mediated almost exclusively by increased expression of ABC transporters (for a review, see reference 26). As expected, all 50 isolates of *C. glabrata*, including the 2 isolates with the petite mutant phenotype, upregulated at least one of the major ABC transporters involved in azole resistance (encoded by *C. glabrata CDR1*, *CDR2*, and *SNQ2*) (5, 22, 23, 27, 29). While the highest levels of gene expression did not always correlate with the highest MICs, it should be noted that fluconazole-SDD and -resistant *C. glabrata* isolates expressed higher constitutive levels of ABC transporters than fluconazole-susceptible isolates; importantly, none of the latter isolates showed expression levels above the threshold value established for significant gene upregulation (see Table S1 in the supplemental material). Among the 11 fluconazole-resistant *C. albicans* isolates, 7 isolates were found to upregulate *CDR* and/or *MDR1* genes and to exhibit the Erg11p amino acid substitutions shown to cause azole resistance (24, 25); for the remaining isolates, two showed *CDR1* and *CDR2* overexpression and two Erg11p mutations. Interestingly, only upregulated expression of multidrug transporters was noted in all of eight fluconazole-SDD *C. albicans* isolates (see Table S1 in the supplemental material), emphasizing the concept of the presence of Erg11p mutations, alone or in combination with other mechanisms, as a contributor to azole resistance in *C. albicans* (10, 13).

In this study, the Vitek 2 system exhibited excellent agreement compared to the reference BMD method, with overall CA of 97.5% for both fluconazole and voriconazole. These values were even higher than those achieved by Pfaller at al., who reported rates of CA for the comparison of the Vitek 2 system results with the 48-h BMD results of 88.3% for fluconazole (18) and 98.2% for voriconazole (19), thus indicating that the Vitek 2 system is a reliable technique for susceptibility testing of azole antifungal agents. The Vitek 2 system was able to identify almost all the azole-resistant organisms but with minor errors, observed for only four isolates, one *C. albicans* isolate and three *C. glabrata* isolates. Our results are comparable to those for other commercial techniques for antifungal susceptibility testing, such as the WIDERYST system (8). Compared to the latter method, the Vitek 2 system has the advantage of performing antifungal susceptibility testing in a fully automated fashion for most species of *Candida* (18, 19).

In conclusion, the Vitek 2 system provides a very promising alternative to reference methods for antifungal susceptibility testing of isolates belonging to the most clinically relevant *Candida* species, thus providing fast and reliable means for detecting azole resistance. However, the available therapeutic options are limited in cases of isolates found to be cross-resistant to azoles (fluconazole and voriconazole). In the future, newly licensed antifungal agents, such as caspofungin, posaconazole, and anidulafungin, should be included in this system in order to improve its clinical usefulness.

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