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Research Paper

Use of the VITEK 2 system to identify and test the antifungal susceptibility of clinically relevant yeast species

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Abstract

Eleven quality control isolates (*Candida albicans* ATCC 64548, *C. tropicalis* ATCC 200956, *C. glabrata* ATCC 90030, *C. lusitaniae* ATCC 200951, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, *C. dubliniensis* ATCC 6330, *Saccharomyces cerevisiae* ATCC 9763, *Cryptococcus neoformans* ATCC 90012, *C. gattii* FIOCRUZ-CPF 60, and *Trichosporon mucoides* ATCC 204094) and 32 bloodstream isolates, including *C. albicans*, *C. tropicalis, C. parapsilosis, C. glabrata, C. krusei*, *C. guilliermondii*, *C. pelliculosa* (*Pichia anomala*), *C. haemulonii, C. lusitaniae, and C. kefyr* were identified at the species level by the VITEK 2 system. A set of clinical isolates (32 total) were used as challenge strains to evaluate the ability of the VITEK 2 system to determine the antifungal susceptibility of yeasts compared with the CLSI and EUCAST BMD reference standards. The VITEK 2 system correctly identified 100% of the challenge strains. The identification of yeast species and the evaluation of their susceptibility profiles were performed in an automated manner by the VITEK 2 system after approximately 15 h of growth for most species of *Candida*. The VITEK 2 system ensures that each test is performed in a standardized manner and provides quantitative MIC results that are reproducible and accurate when compared with the BMD reference methods. This system was able to determine the MICs of amphotericin B, flucytosine, voriconazole, and fluconazole in 15 h or less for the most common clinically relevant *Candida* species. In addition, the VITEK 2 system could reliably identify resistance to flucytosine, voriconazole, and fluconazole and exhibits excellent quantitative and qualitative agreement with the CLSI or EUCAST broth microdilution reference methods.

Key words: antifungal agents, *Candida*, susceptibility testing methods, MIC, CLSI, EUCAST.

Introduction

The incidence of invasive nosocomial fungal infections caused by yeasts has been significantly increasing over the last two decades, resulting in high morbidity and mortality (Espinel-Ingroff *et al.*, 2005). Although *Candida albicans* is the primary etiological agent of candidemia worldwide, non-*C. albicans* species are likely to overtake *C. albicans* in some hospital units, particularly those in Brazilian hospitals (Perkins *et al.*, 2005). Among the non-*C. albicans* species, *C. tropicalis, C. parapsilosis* and *C. glabrata* are the most common causative agents of candidemia in Brazil (Meyer *et al.*, 2009). The prognosis of candidemia depends on the immunological status of the host, on the virulence of the fungal species, on the resistance of the causative strain to antifungals and on the efficacy of antifungal therapy (Pereira *et al.*, 2010). *Candida glabrata* is known to have acquired resistance to fluconazole and other azole drugs. Intrinsic resistance to older azoles has been well established for *C. krusei*, and primary resistance to amphotericin has been described in species, such as *C. lusitaniae* and *C. haemulonii* (Rodriguez-Tudela *et al.*, 2008). Concern about antifungal resistance, particularly with azole-class agents, amphotericin B and echinocandins, a new class of antifungal, necessitates the accurate *in vitro* susceptibility testing of medically relevant yeasts (Clinical and Laboratory Standards Institute, 2008; Diekema *et al.*, 2009).

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The susceptibility patterns of *Candida* spp. are often unknown, and empiric therapy is frequently used to treat infections. Early species identification and rapid antifungal susceptibility testing (AFST) are needed in cases of critical infections. Standardized broth microdilution methods for AFST, published by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST), have served as the standard of reference for AFST (Rodriguez-Tudela *et al.*, 2008; Verweij *et al.*, 1999).

Although a number of AFST systems have been developed and are commercially available, their performance is variable (Buchaille *et al.*, 1998; Cuenca-Estrella *et al.*, 2005; Hata *et al.*, 2007; Zaragoza *et al.*, 2011). The VITEK 2 system (bioMérieux, Inc., Hazelwood, MO) is a fully automated system that permits both species identification and antifungal susceptibility testing. (Pfaller *et al.*, 2007; Revankar *et al.*, 1998). The aim of this study is to evaluate the commercial VITEK 2 system for species identification and antifungal susceptibility testing of Brazilian bloodstream isolates. The minimum inhibition concentration (MIC) results obtained using the VITEK 2 system were compared with those obtained using the CLSI and EUCAST reference procedures. For the CLSI method, results were calculated at both 24 and 48 h of incubation. Agreement between the antifungal drug susceptibility results obtained using the VITEK 2 system and those obtained using each BMD method ($\pm$ 2 $\log_2$ dilutions) was assessed by combining the data obtained from both the quality control and from the challenge clinical isolates in all tests (Cuenca-Estrella *et al.*, 2010).

Materials and Methods

Study design

The purpose of this study was to evaluate the utility of the commercially available VITEK 2 system for the identification of medical yeasts at the species level. This study was also designed to compare the MIC results obtained by the VITEK 2 method for fluconazole, voriconazole, amphotericin B, and flucytosine to those obtained by the CLSI and EUCAST BMD methods (Clinical and Laboratory Standards Institute, 2008; Verweij, 1999). Furthermore, this study uses the recent recommendations published by EUCAST for the testing of non-fermentative yeasts, *e.g.*, *Cryptococcus.* and *Trichosporon* species. The intralaboratory reproducibility of the identification tests and of the antifungal susceptibility tests was determined by testing all isolates in triplicate on three separate days. The MIC results obtained using the VITEK 2 system following 10 to 30 h of incubation (depending on the growth rate of the organism) were compared to those obtained using the CLSI reference BMD method after 24 and 48 h of incubation and using the EUCAST BMD method after 24 h of incubation (Cuenca-Estrella *et al.*, 2010).

Test organisms

The test organisms evaluated in this study included 11 American Type Culture Collection (ATCC) strains that have been established as QC strains for species identification tests: *C. albicans* ATCC 64548, *C. tropicalis* ATCC 200956, *C. glabrata* ATCC 90030, *C. lusitaniae* ATCC 200951, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, *C. dubliniensis* ATCC 6330, *Saccharomyces cerevisiae* ATCC 9763, *C. neoformans* ATCC 90012, *C. gattii* FIOCRUZ-CPF 60, and *Trichosporon mucoides* ATCC 204094. The challenge strains used for the species identification and antifungal susceptibility tests were bloodstream clinical isolates (n = 32) of *C. albicans* (n = 5), *C. tropicalis* (n = 5), *C. parapsilosis* (n = 5), *C. glabrata* (n = 5), *C. krusei* (n = 5), *C. guilliermondii* (n = 3), *C. pelliculosa (Pichia anomala)* (n = 1), *C. haemulonii* (n = 1), *C. lusitaniae* (n = 1), and *C. kefyr* (n = 1). These strains are all recent clinical isolates and were selected to represent clinically prevalent fungal species, including fluconazole-resistant strains and amphotericin B-resistant species. These isolates were selected to provide on-scale azole MICs ranging from 0.12 - > 64 mg/L, voriconazole MICs from 0.06 to 4 mg/L, flucytosine MICs from 0.015-16 mg/L, and amphotericin B MICs from 0.015-4 mg/L. All isolates were previously identified by the API 20C AUX test (bioMérieux) or by conventional methods (Matta *et al.*, 2007).

Identification tests

Prior to testing, a suspension of each isolate was inoculated at least twice onto Can 2 chromogenic agar plates (bioMérieux, France) and onto Sabouraud dextrose agar slants to ensure the purity and the viability of the cultures. The inoculum suspensions for the VITEK 2 were prepared in sterile saline at a turbidity equal to a 2.0 McFarland standard, as measured using a DensiChek instrument (bioMérieux). The individual test cards were automatically filled with the prepared culture suspension, sealed, and incubated by the VITEK 2 instrument. The cards were incubated at 35.5 °C for 18 h, and optical density readings were taken automatically every 15 min. The final profile results were compared with the database, and the identification of the unknown organism was obtained.

A final identification of "excellent," "very good," "good," "acceptable," or "low- discrimination" was considered to be correct. The clinical isolates were previously identified at the species level by the API 20C AUX system (bioMérieux), which utilizes 20-cupule plastic strips containing dehydrated carbohydrate substrates. Conventional plate assimilation tests (auxanograms) for carbon use were also performed on these isolates using the following 27 compounds: glucose, L-sorbose, galactose, D-ribose, D-xylose, L-arabinose, D-arabinose, L-rhamnose, salicin, melibiose, sucrose, alpha-methil-D-glucoside, inositol, maltose, trehalose, cellobiose, lactose, raffinose, D-glu-

citol, ribitol, erythritol, glycerol, melezitose, D-mannitol, galactitol, D-L lactic acid, and D-L-succinic acid. Plate assimilation tests for nitrogen use were performed with peptone and potassium nitrate. In addition, yeast isolates were evaluated for fermentation using Durham tubes and for urease activity. Filamentous growth and chlamydospore production were assessed on corn meal agar (Matta *et al.*, 2007).

Both the conventional tests and the tests performed using the API 20 C AUXA were considered to be reference standards for this study, as both approaches were able to correctly identify the clinical isolates at the species level. The two approaches were evaluated independently, and personnel were blinded to the identity of the isolates.

Antifungal agents

The VITEK 2 cards containing serial twofold dilutions of amphotericin B, fluconazole, flucytosine, and voriconazole were provided by the manufacturer. For this study, microdilution plates containing serial twofold dilutions of each drug were prepared according to the CLSI or EUCAST BMD recommendations. The following antifungal compounds were included in the BMD assay: amphotericin B (0.03-16 μg/mL, Sigma-Aldrich), flucytosine (0.12-64 μg/mL, Sigma-Aldrich), fluconazole (0.12-64 μg/mL, Pfizer S.A., NY), and voriconazole (0.015-8 μg/mL, Pfizer S.A., NY). A stock solution of each antifungal agent was prepared in two-milliliter aliquots in either dimethyl-sulfoxide (amphotericin B and voriconazole) or in distilled water (fluconazole and flucytosine). The media used for the final drug dilutions was RPMI 1640 with potassium bicarbonate and without L-glutamine, buffered to pH 7 using 165 mM MOPS buffer (Sigma-Aldrich). The media were prepared as 2x stocks, and 100 μL was added to each well of the microdilution plates. The plates were sealed and were stored at -80 °C until use.

Antifungal susceptibility testing (AST)

Yeast inocula were prepared in sterile distilled water from a 24-h (*Candida* spp.) or from a 48-h culture (*Cryptococcus* spp.) and were incubated on Sabouraud dextrose agar at 35 °C or 30 °C (*C.* spp.). The inocula for the VITEK 2 were prepared in sterile saline to a turbidity equal to a 2.0 McFarland standard according to a bioMérieux DensiChek instrument. Each standardized inoculum suspension was placed into a VITEK 2 cassette along with a sterile polystyrene test tube and a yeast susceptibility test card. The cassettes were placed in the VITEK 2 instrument and the respective yeast suspensions were diluted appropriately, after which the cards were filled, incubated, and read automatically by the VITEK 2. The time of incubation varied from 10 to 30 h based on the growth rate in the drug-free control well, and the results were expressed as MICs in micrograms per milliliter.

The inocula for the BMD reference assays were prepared by diluting a portion of the suspension that was prepared for the VITEK 2 assay to a turbidity equivalent to a 0.5 McFarland standard. The turbidity of each inoculum was measured spectrophotometrically, and suspensions of 10^6 cfu/mL were prepared. These suspensions were further diluted to yield a final concentration for each inoculum, as indicated by the CLSI document M27-A3 (1-5 x 10^3 cfu/mL) or by the EUCAST (1-5 x 10^5 cfu/mL) BMD method. The final inocula were inoculated in the wells of the BMD plates containing antifungals. The plates were incubated at 35 °C (*Candida* spp.) or at 30 °C (*C.* spp.) for up to 48 h. The plates prepared by the EUCAST BMD method were read at 24 h of incubation, and those prepared by the CLSI BMD method were read at both 24 and 48 h of incubation. The optical density (OD) of each well was assessed spectrophotometrically (Titertek Multiskan, Sweden) at 492 nm. MIC values for fluconazole, voriconazole, and flucytosine were defined as the lowest concentration of antifungal that resulted in a 50% reduction in the OD relative to the drug-free control. For amphotericin B, the MIC was defined as the concentration of antifungal that reduced growth by 100% (CLSI method) or by 90% (EUCAST method) compared with the control (3, 23, 25). *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were included in all VITEK 2 and CLSI or EUCAST BMD assays to provide on-scale MIC results and to represent fluconazole-susceptible and fluconazole-resistant species, respectively. In this study, all MICs by strain were in their respective reference ranges.

Analysis of results

The reproducibility and accuracy of the results obtained using the VITEK 2 system compared to the reference techniques were calculated to determine a percentage of essential agreement (A) between the species identification and the MIC values. Discrepancies among MIC endpoints of more than ± 2 $\log_2$ dilutions were used to calculate the essential agreement (Cuenca-Estrella *et al.*, 2010; Pfaller *et al.*, 2007).

Results

All eleven type strain isolates and all 32 bloodstream clinical isolates demonstrated sufficient growth at 15 h of incubation, and the species could be determined by the VITEK 2 system at different incubation periods. The VITEK 2 system met the desired reproducibility criteria (100% correct) for the identification of all QC strains at the species level. Two of the 32 clinical isolates tested (*C. guilliermondii* and *C. glabrata*) yielded low-discrimination (but correct) results and required supplemental observation and testing to resolve their identities to a single taxon. Following additional tests, as recommended by the manufacturer, all 32 challenge strains were successfully identified

by the VITEK 2 system. No isolates failed to grow in the VITEK 2 system in any of the replicates in any test.

All eleven type strain isolates and thirty one bloodstream clinical isolates were correctly identified at the species level by the VITEK 2 system. One *Cryptococcus* isolate that was recently reclassified by molecular methodologies as a new species - *C. gattii* - was identified as *C. neoformans*. The two *Cryptococcus* type strains and the *Trichosporon mucoides* type strain exhibited the characteristics of slow-growing organisms.

To assess the performance of the VITEK 2 system for the determination of MICs, we evaluated 32 clinical isolates. The VITEK 2 MIC results were produced after 14 to 27 h of incubation for all *Candida* spp. The mean time-to-result for the VITEK 2 system was 15 h for amphotericin B (with a range of 11 to 27.8 h) and fluconazole (with a range of 9 to 24.2 h), 13.1 h for flucytosine (with a range of 13.0 to 27 h), and 12.4 h for voriconazole (with a range of 8.1 to 25.1 h). All except 7 of the 32 clinical isolates (5 isolates of *C. krusei*, *C. kefyr*, and *C. pelliculosa*) demonstrated sufficient growth at 24 h of incubation for the CLSI BMD method, allowing the majority of the MICs to be determined at this time point.

Table 1 summarizes the *in vitro* susceptibilities of 32 bloodstream clinical isolates of *Candida* spp. to amphotericin B, 5-flucytosine, fluconazole and voriconazole, as determined by the VITEK 2 system and by the two reference BMD methods (CLSI and EUCAST). For the CLSI method, the 24-h results are presented for all but seven isolates, which did not exhibit sufficient growth at this point; for these isolates, the results from 48 h are presented.

Table 1 - Antifungal susceptibilities of 32 bloodstream clinical isolates of *Candida* spp. as determined by the VITEK 2 system and the two reference CLSI and EUCAST BMD methodologies.

Species	MIC (mg/L)				
	Antifungal agent	Test method	Range	MIC 50	A(%)*
C. albicans (5)	fluconazole	VITEK 2	≤ 1		
		CLSI	0.12-0.25	0.12	100
		AFST-EUCAST	0.25-0.5	0.25	100
	voriconazole	VITEK 2	≤ 0.12		
		CLSI	0.015-0.25	0.015	100
		AFST-EUCAST	0.015-0.5	0.03	100
	flucytosine	VITEK 2	≤ 1		
		CLSI	0.12-0.25	0.12	100
		AFST-EUCAST	0.25-0.5	0.25	100
	amphotericin B	VITEK 2	≤ 0.25-1	0.5	
		CLSI	0.12-0.5	0.25	100
		AFST-EUCAST	0.08-0.25	0.06	100
C. tropicalis (5)	fluconazole	VITEK 2	≤ 1		
		CLSI	0.12-0.5	0.12	100
		AFST-EUCAST	0.25-1	0.25	100
	voriconazole	VITEK 2	≤ 0.12		
		CLSI	0.015-0.6	0.06	100
		AFST-EUCAST	0.03-0.12	0.03	100
	flucytosine	VITEK 2	≤ 1		
		CLSI	0.12-2	0.12	100
		AFST-EUCAST	0.25-4	0.25	100
	amphotericin B	VITEK 2	≤ 1		
		CLSI	0.12-0.5	0.25	100
		AFST-EUCAST	0.25-1	0.12	100
C. glabrata (5)	fluconazole	VITEK 2	2 - ≥ 64	4	
		CLSI	1 - ≥ 64	1	50
		AFST-EUCAST	1 - ≥ 64	1	50
	voriconazole	VITEK 2	≤ 0.12-1	0.12	
		CLSI	0.06-4	0.12	100
		AFST-EUCAST	0.12-4	0.12	100

Table 1 (cont.)

Species	MIC (mg/L)				
	Antifungal agent	Test method	Range	MIC 50	A(%)*
	flucytosine	VITEK 2	≤ 1		
		CLSI	0.12-0.5	0.12	100
		AFST-EUCAST	0.25-0.5	0.25	100
	amphotericin B	VITEK 2	0.5-1	0.5	
		CLSI	0.5-1	0.5	100
		AFST-EUCAST	0.12-0.5	0.25	100
C. krusei (5)	fluconazole	VITEK 2	16-32	16	
		CLSI	16-32	32	100
		AFST-EUCAST	16-32	32	100
	voriconazole	VITEK 2	≤ 0.12-0.25	0.25	
		CLSI	0.12-0.5	0.5	100
		AFST-EUCAST	0.015-0.5	0.25	100
	flucytosine	VITEK 2	8.0-16	16	
		CLSI	4.0-16	8	100
		AFST-EUCAST	8	8	100
	amphotericin B	VITEK 2	0.5-4	0.5	
		CLSI	1	1	100
		AFST-EUCAST	0.5-2	0.5	100
C. parapsilosis (5)	fluconazole	VITEK 2	≤ 1-4	≤ 1	
		CLSI	0.12-0.5	0.12	80
		AFST-EUCAST	0.12-1	0.5	100
	voriconazole	VITEK 2	≤ 0.12-0.25	≤ 0.12	
		CLSI	0.12-0.5	0.15	100
		AFST-EUCAST	0.015-0.5	0.06	100
	flucytosine	VITEK 2	≤ 1	≤ 1	
		CLSI	0.12	0.12	100
		AFST-EUCAST	0.25	0.25	100
	amphotericin B	VITEK 2	≤ 0.25-0.5	≤ 0.25	
		CLSI	0.12-0.5	0.25	100
		AFST-EUCAST	0.06-0.5	0.12	100
C. lusitaniae (1)	fluconazole	VITEK 2	≤ 1		
		CLSI	0.12		100
		AFST-EUCAST	0.25		100
	voriconazole	VITEK 2	≤ 0.12		
		CLSI	0.015		100
		AFST-EUCAST	0.03		100
	flucytosine	VITEK 2	≤ 1		
		CLSI	0.12		100
		AFST-EUCAST	0.25		100
	amphotericin B	VITEK 2	≥ 16		0
		CLSI	2		0
		AFST-EUCAST	2		
C. dubliniensis (1)	fluconazole	VITEK 2	≤ 1		
		CLSI	0.25		100
		AFST-EUCAST	0.12		100
	voriconazole	VITEK 2	≤ 0.12		
		CLSI	0.015		100

Table 1 (cont.)

Species	MIC (mg/L)				
	Antifungal agent	Test method	Range	MIC 50	A(%)*
		AFST-EUCAST	0.03		100
	flucytosine	VITEK 2	≤ 1		
		CLSI	0.12		100
		AFST-EUCAST	0.25		100
	amphotericin B	VITEK 2	≤ 0.25		
		CLSI	0.25		100
		AFST-EUCAST	0.12		100
C. haemunolii (n=1)	fluconazole	VITEK 2	≥ 64		
		CLSI	0.12		0
		AFST-EUCAST	0.12		0
	voriconazole	VITEK 2	1		
		CLSI	0.015		0
		AFST-EUCAST	0.03		0
	flucytosine	VITEK 2	≤ 1		
		CLSI	0.25		100
		AFST-EUCAST	0.5		100
	amphotericin B	VITEK 2	≥ 16		
		CLSI	8		100
		AFST-EUCAST	4		100
C. kefyr (n=1)	fluconazole	VITEK 2	2		
		CLSI	0.5		100
		AFST-EUCAST	0.5		100
	voriconazole	VITEK 2	≤ 0.12		
		CLSI	0.03		100
		AFST-EUCAST	0.03		100
	flucytosine	VITEK 2	≤ 1		
		CLSI	0.12		100
		AFST-EUCAST	0.12		100
	amphotericin B	VITEK 2	2		
		CLSI	0.25		0
		AFST-EUCAST	0.25		0
C. pelliculosa (n = 1)	fluconazole	VITEK 2	2		
		CLSI	2		100
		AFST-EUCAST	2		100
	voriconazole	VITEK 2	≤ 0.12		
		CLSI	0.25		100
		AFST-EUCAST	0.25		100
	flucytosine	VITEK 2	≤ 1		
		CLSI	0.12		100
		AFST-EUCAST	0.25		100
	amphotericin B	VITEK 2	≤ 0.25		
		CLSI	0.12		100
		AFST-EUCAST	0.12		100

*A, agreement (±2 Log 2 dilutions) between VITEK and broth microdilution method MICs.

Discussion

All eleven quality control isolates were successfully identified using the VITEK 2 system. Quality control tests to assess the accuracy and reproducibility of this system yielded the expected results, covering the range of identifiable species according to the manufacturer's claims. Using clinical samples, the overall agreement between the VITEK 2 system and the reference methods (*i.e.*, the conventional methods and the API 20 C AUX) upon initial testing was 93.6% (29/31). In our study, two (6.5%) low-discrimination identification results were obtained for *C. guilliermondii* and *C. glabrata*. Additional tests revealed that the identification of these two clinical isolates was correct, thus yielding the correct identification for all the clinical isolates tested.

When the results of the reference and challenge tests were combined, the VITEK 2 system correctly identified a total of 42 isolates (11 reference isolates and 31 challenge isolates) at the species level. Hata *et al.* (2007) discovered an error in identification using the VITEK 2 system for one clinical isolate of *C. glabrata*. However, we did not observe any misidentification or low-discrimination results for *C. glabrata* or even for *C. krusei*, *C. parapsilosis*, and *C. tropicalis* (Hazen and Howell, 2003). In fact, the VITEK 2 system performed satisfactorily in the identification of non-*Candida* yeast species; isolates of *Saccharomyces cerevisiae* and *Trichosporon mucoides* were accurately identified by this system. Notably, *Trichosporon* species are somewhat refractory to the preparation of a uniform suspension in saline, leading to inaccurate results. A careful technique is required to achieve a homogeneous McFarland 2.0 turbidity, increasing the reproducibility of the results regardless the method employed.

The fact that *C. gattii* was not identified by the VITEK 2 system was expected, as this species is not present in the system database. The identification of this new species (formerly, *C. neoformans* var. *gattii*) requires additional molecular approaches (Pappas *et al.*, 2004). Therefore, the identification of *C. gattii*, as *C. neoformans* is acceptable for this phenotypic approach; technical laboratory staff should be aware of this discrepancy and should submit clinical isolates to the appropriate reference center for genetic analysis as required. In this study, the identification of *C.* at the genus level using the VITEK 2 system was considered useful, as *C.* spp. cause serious, life-threatening meningeal cryptococcal infections. *In vitro* resistance to antifungals exhibited by members of this medically relevant genus is a concern among physicians (Pfaller *et al.*, 2007). Although we could not determine MIC values for *C. neoformans* or *C. gattii* isolates due to insufficient growth at 30 h, other authors have successfully employed this system to assess the susceptibility profile of *C. neoformans* strains (Cuenca-Estrella *et al.*, 2010). From a clinical laboratory perspective, extensive work should be performed to determine the MIC profile for *C. neoformans* isolates such that these strains can be included in the VITEK 2 system database, which will improve its clinical utility.

In addition, we confirmed a clinically important advantage of the VITEK 2 system over conventional identification methods and over the API 20C AUXA system: species were identified within 18 h compared to 48-72 h for the other methods (Hazen *et al.*, 2003).

The fluconazole MIC results obtained using the VITEK 2 system were highly reproducible, as determined by testing all 32 clinical isolates in triplicate on three different days (data not shown). The intralaboratory reproducibility of these results was 100% for all organisms tested, demonstrating the high level of standardization achieved using this automated microbiology system, which eliminates the subjectivity that could affect other approaches lacking this automation. The use of a spectrophotometer to provide objective readings of MICs at early time points was thoroughly established by the EUCAST committee and can also be employed for the CLSI BMD method (Clinical and Laboratory Standards Institute, 2008; Pfaller *et al.*, 2007; Verweij *et al.*, 1999).

Others have reported that the VITEK 2 system was able to determine fluconazole MIC endpoints after 9.1 to 27.1 h of incubation (mean, 12 to 14 h). In this study, we confirmed this finding and observed a 15-h median MIC endpoint for fluconazole. The determination of MIC values for *C. neoformans*, *C. gattii* and *Trichosporon* spp. by the VITEK 2 system required an incubation period greater than 30 h, in agreement with a previous report (Cuenca-Estrella *et al.*, 2010). It noteworthy that the VITEK 2 system was able to determine the MIC endpoints earlier than the BMD method, especially for *C. tropicalis* isolates, which required 18 h of incubation, as previously noted (Pfaller *et al.*, 2007).

In this study, we confirm that the VITEK 2 system is able to reliably identify the high flucytosine, voriconazole, and fluconazole MICs that are characteristic of resistant fungal isolates. Recently, Pfaller *et al.* (2007) have shown that fluconazole may serve as a surrogate marker for voriconazole susceptibility in *Candida* spp. Elevated fluconazole MICs ([#GTEQ#] 64 mg/L) predict the resistance of *Candida* spp. to voriconazole with an absolute categorical agreement of 97%, 0.1% very major errors, and 1.4% major errors (Posteraro *et al.*, 2009). Therefore, testing for fluconazole susceptibility in a hospital setting using the VITEK 2 system can function as a surrogate test for voriconazole resistance.

The antifungal resistance results for the 32 clinical isolates are presented in Table 1. Fluconazole MICs for 25 clinical isolates at 24 h, as determined by the CLSI BMD method were compiled for the agreement analysis, which was performed to reduce the likelihood of falsely elevated fluconazole MICs due to trailing growth (Clinical and Laboratory Standards Institute, 2008). Furthermore, previous work has shown that the fluconazole MIC value at 24 h cor-

related better with the clinical outcome and with sterol quantification than the MIC value at 48 h (1, 20). The MICs obtained using the VITEK 2 system were somewhat higher than those observed for the two reference methods. This finding may be because the range of antifungal concentrations used in this system does not match exactly the range used in the other techniques. In the case of fluconazole, for example, the reference method concentration range was 0.12 to 64 mg/L, while the VITEK 2 concentration range was 1 to 64 mg/L).

The overall agreement between the results obtained using the VITEK 2 system, and those obtained using the CLSI and EUCAST BMD reference methods were high (88.5%). In recent studies, the VITEK 2 system has demonstrated a high level of reproducibility and an excellent categorical agreement with the CLSI microdilution reference procedure for fluconazole (at 95%) and, more recently, for amphotericin B, flucytosine, and voriconazole (Cuenca-Estrella *et al.*, 2010; Pfaller *et al.*, 2007; Revankar *et al.*, 1998).

Table 1 shows the agreement rates between procedures by antifungal agent. The rates of agreement were high when the results were analyzed per species. Regarding the individual species of *Candida*, the A between the VITEK 2 results and either of the BMD MICs was 100% for *C. albicans, C. tropicalis, C. dubliniensis, C. pelliculosa* (*Pichia anomala*), and *C. guilliermondii.* The A value is maximal for nearly all clinically relevant species except *C. parapsilosis* and *C. glabrata*, for which the A values were 97.5 and 87.5%, respectively, between the results obtained using the VITEK 2 system and those obtained by the reference methods for all four antifungal drugs. For *C. glabrata*, the relatively poor agreement was due to the significantly (four-fold) higher fluconazole MICs for two isolates, as measured by the VITEK 2 system, compared to those obtained by both the CLSI and the EUCAST BMD methods. The agreement calculated for the fluconazole MIC in this study was lower (A, 50%) than previously described (A, 89.2%) by Pfaller *et al.* (2007). Given that *C. glabrata* is a species with known fluconazole resistance, these discrepancies should not pose a problem clinically.

Similar discrepancies were observed between the VITEK 2 system and the CLSI BMD method for one *C. parapsilosis* isolate. Notably, all three isolates (two *C. glabrata* and one *C. parapsilosis* isolate) exhibited trailing phenotypes, which may have a significant influence on the MIC determination by the VITEK 2 system for these two species. In this study, the trailing phenomenon, a well-known technical artifact observed with fungistatic drugs, occurred in all *C. tropicalis* isolates and in the majority of *C. glabrata* and *C. albicans* isolates, regardless of the BMD method used. Trailing is a recognized factor that contributes to false positives for antifungal resistance, as this phenomenon results in residual fungal growth above the MIC endpoint. For this reason, MIC readings are typically measured at 50% growth inhibition compared with the positive control. We observed that the VITEK 2 system, despite measuring fungal growth spectrophotometrically, yielded correct results even for the trailing phenotype frequently observed in *C. tropicalis* isolates.

In addition, the amphotericin B MICs generated by the VITEK 2 were higher than those obtained by the BMD method for *C. lusitaniae* and *C. kefyr*, and for *C. haemulonii*, another uncommon fungal species, the fluconazole and voriconazole MICs generated by the VITEK 2 system were fivefold higher than those obtained using the BMD method. These discrepancies emphasize the impact of trailing on BMD results obtained with these uncommon species and suggest that future studies will be required to better evaluate these emergent bloodstream isolates (Meyer *et al.*, 2009; Rodriguez-Tudela *et al.*, 2008). Notably, although we analyzed a smaller number of *C. glabrata* isolates, we did not observe a discrepancy in the voriconazole MIC for this species, as previously reported (Pfaller *et al.*, 2007).

The only false-positive resistance results for the antifungal agent amphotericin B were observed with *C. lusitaniae* and *C. kefyr*. *C. lusitaniae* is a rare, emergent species of clinical concern due to its potential to develop resistance *in vitro* to amphotericin B. The high MIC value for *C. lusitaniae*, as determined by the VITEK 2 system, was not confirmed by the CLSI or EUCAST BMD methods, suggesting that a greater number of *C. lusitaniae* isolates will need to be evaluated to confirm our findings. Moreover, the artificially elevated fluconazole and voriconazole MIC values for this species were obtained using the VITEK 2 system, emphasizing the need to study this uncommon species in greater detail to confirm our observations and to contribute to a standardized test.

Interestingly, using the VITEK 2 system we observed a high amphotericin B MIC for a single clinical isolate of *C. haemulonii* that was also observed using the reference methods, confirming the resistance of this species to amphotericin B.

In this study of clinical bloodstream fungal isolates from Brazil, we confirm that the VITEK 2 system used to identify fungal species and to determine antifungal susceptibility is in excellent agreement with the reference BMD methods. The ability of the VITEK 2 system to provide quantitative MIC results is reproducible and accurate, identifying high MIC values that indicate resistance to flucytosine, voriconazole, and fluconazole. The MICs of fluconazole, voriconazole, amphotericin B, and flucytosine could be determined rapidly (~15 h) for most species of *Candida* using this system. The monitoring of the development of antifungal resistance during therapy should be assessed by AFST on a routine basis. The availability of rapid antifungal susceptibility data may play a major role in optimizing the therapy of invasive candidal infections. Furthermore, surveillance of antifungal susceptibility profiles pro-

vides a useful tool for hospitals to validate empiric treatment regimens.

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