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Wild-Type MIC Distributions and Epidemiological Cutoff Values for Fluconazole, Posaconazole and Voriconazole versus *Cryptococcus neoformans* as Determined by 72 hour CLSI Broth Microdilution Method

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ABSTRACT

Background: When clinical susceptibility breakpoints (CBPs) are absent, establishing wild-type (WT) MIC distributions and epidemiological cutoff values (ECVs) provides sensitive means for detecting emerging resistance. We determined species-specific ECVs for fluconazole (FLC), posaconazole (PSC) and voriconazole (VRC) using a large global collection of *C. neoformans* (CNEO) isolates obtained from the ARTEMIS and SENTRY Antimicrobial Surveillance Programs.

Methods: From 2006-2009, 285 invasive clinical isolates of CNEO were collected from 61 centers worldwide (178 isolates from ARTEMIS and 107 from SENTRY Program) and susceptibility testing was performed against FLC, PSC and VRC using CLSI M27-A3 broth microdilution method (72-h incubation). ARTEMIS isolates were tested at the University of Iowa and SENTRY Program isolates were tested at JMI Laboratories and the results were combined for analysis. An additional collection of 986 isolates tested against FLC between 1996 and 2008 were used to assess temporal trends in the frequency of non-WT isolates.

Results: The modal MICs (µg/ml) for FLC, PSC and VRC, respectively, were: 4, 0.12 and 0.06. The ECVs expressed as µg/ml (% of isolates that had MIC ≤ ECV) for FLC, PSC and VRC, respectively, were: 8 (96.9), 0.25 (96.5) and 0.12 (95.1). Temporal trends in the emergence of non-WT strains (% of isolate MICs > ECV) for the time periods 1996-2000, 2001-2004, 2005-2008, respectively, for FLC were 4.2, 3.8 and 0.5.

Conclusions: In the absence of CBPs for FLC, PSC and VRC these WT MIC distributions and ECVs will be useful in surveillance for emergence of azole reduced susceptibility among CNEO. Application of the FLC ECV to a large collection of CNEO tested (1996-2008) revealed a decrease in the frequency of non-WT strains over time. These findings are consistent with those of more limited surveys in developed countries suggesting that CNEO susceptibility to FLC has improved since the introduction of antiretroviral therapy. Continued surveillance using these ECVs for the azoles and CNEO is warranted.

INTRODUCTION

Cryptococcus neoformans is the most common species of *Cryptococcus* affecting patients with AIDS and other immunocompromising conditions. The Clinical and Laboratory Standards Institute (CLSI) Subcommittee for Antifungal Testing has developed standardized broth microdilution (BMD) and disk diffusion (DD) methods for in vitro susceptibility testing of *C. neoformans* against fluconazole and other azoles. Although there were some early concerns that the CLSI method was not optimal for testing *C. neoformans*, the CLSI BMD and DD methods have now been applied worldwide to: (i) study the emergence of fluconazole resistance in clinical isolates of *C. neoformans*; (ii) provide a comparison of the activity of new and established agents against *C. neoformans*; (iii) identify geographic and temporal trends in antifungal resistance, and (iv) identify clinical and laboratory strains that can be used to study mechanisms of resistance.

Despite the availability of these standardized methods, MIC and zone diameter interpretive breakpoints have not been established for any antifungal agent against *C. neoformans*.

In the present study, we used the MIC distributions for fluconazole, posaconazole, and voriconazole tested against *C. neoformans* isolates from two independent antifungal surveys, the ARTEMIS and SENTRY Antimicrobial Surveillance Programs, for the 3-year time period 2006-2008 to establish epidemiologic cut off values (ECVs) for each agent. We then applied the ECVs for each azole to a larger global collection of 986 clinical isolates of *C. neoformans* collected from more than 100 medical centers and testing using CLSI BMD methods to determine the frequency of reduced susceptibility (emergence of non-wild-type [WT] strains) to each agent in four geographic regions over the period 1996-2008.

MATERIALS AND METHODS

Organisms: A total of 285 isolates of *C. neoformans* obtained from 61 medical centers in 23 countries in the ARTEMIS (178 isolates) and SENTRY Program (107 isolates) were tested (Table 1). The isolates represented consecutive incident isolates from patients with either cryptococcal meningitis or cryptococcemia (one isolate per patient) collected at the respective medical centers between 2006 and 2008. These results were used to establish the ECVs for each antifungal agent.

A second collection of 986 isolates of *C. neoformans* were obtained from more than 100 medical centers during the course of the ARTEMIS global surveillance conducted by the University of Iowa (Iowa City, Iowa, USA) between 1996 and 2008. The MIC distributions for these isolates were assessed using the ECVs for each antifungal agent to determine the frequency of reduced susceptibility to each agent over time and in specific geographic regions.

The isolates were identified by standard methods and stored as water suspensions until used in the study. Prior to being tested, each isolate was passaged at least twice onto potato dextrose agar and CHROMagar *Candida* medium (Becton Dickinson and Company, Sparks, Maryland USA) to ensure purity and viability.

Antifungal susceptibility testing: BMD testing was performed in accordance with the guidelines in CLSI document M27-A3, using RPMI 1640 medium, an inoculum of 0.5 to 2.5 X 10^3 cells/ml, and incubation at 35ºC. MICs were determined visually, after 72h of incubation, as the lowest concentration of drug that caused a significant diminution (≥50% inhibition) of growth relative to that of the growth control.

Quality control (QC) was performed by testing CLSI-recommended strains *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019. All QC results were within published ranges.

Definitions: The definitions of WT organisms and ECVs were those outlined previously. A WT organism is defined as a strain which does not harbor acquired resistance to the particular antimicrobial agent being examined. The typical MIC distribution for WT organisms covers three to five two-fold dilution steps surrounding the modal MIC. Inclusion of WT strains in the present study was ensured by testing only the incident isolate for each infection episode.

The ECVs for fluconazole, posaconazole and voriconazole and *C. neoformans* were obtained as described previously, by considering the WT MIC distribution, the modal MIC for each distribution, and the inherent variability of the test (usually within one $\log_2$ dilution step). In general, the ECV should encompass at least 95% of isolates in the WT distribution. Organisms with acquired resistance mechanisms may be included among those for which the MIC values are higher than the ECV.

RESULTS

- The WT MIC distributions for fluconazole, posaconazole and voriconazole and *C. neoformans* are shown in Table 1. These distributions clearly demonstrate the increased potency of both posaconazole and voriconazole as compared to that of fluconazole (MIC_{90} values, 8, 0.25 and 0.12 µg/ml, respectively).
- The modal MIC values (percentages of isolates with a MIC equal to the mode; Table 1) at 72-h of incubation for fluconazole, posaconazole and voriconazole, respectively, were as follows (Table 1): 4 (49.1%), 0.12 (35.8%) and 0.06 µg/ml (57.2%).
- The modal fluconazole MIC remained unchanged at 4 µg/ml over time and in all geographic regions, the proportion of MIC results that exceeded the ECV (non-WT strains) progressively decreased from 4.2% in 1996-2000 to only 0.5% in 2005-2008 (Table 2).
- Resistance to fluconazole was less common among the more recent (2005-2008) isolates of *C. neoformans* from Asia-Pacific, Europe and North America (0.0% for all three) compared to that observed in the Latin American region (1.6%; Table 2).
- Application of the ECVs for posaconazole or voriconazole MIC distributions from 2001-2008 demonstrates a decrease in non-WT strains over time for both of these alternative azole antifungal agents (Table 3).
- In contrast to what was observed with fluconazole, decreased susceptibility to both posaconazole and voriconazole appeared to be emerging in the Asia-Pacific region (5.1 to 6.3%).

Table 1. Wild-type MIC distributions and ECVs of fluconazole, posaconazole and voriconazole for 285 *Cryptococcus neoformans* strains obtained using CLSI reference broth microdilution methods[a].

Antifungal agent	No. of isolates with MIC (µg/ml):													MIC (µg/ml)		ECV (µg/ml [%])[b]
	≤0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	Range	Mode	
Fluconazole						3	2	24	62	140	45	8	1	0.25-32	4	8 (96.9)
Posaconazole			12	100	102	61	10							0.03-0.5	0.12	0.25 (96.5)
Voriconazole	1	15	48	163	44	13	1							0.008-0.5	0.06	0.12 (95.1)

a. MIC values determined according to CLSI document M27-A3 with an incubation time of 72h.
b. Percentage of isolates for which the MIC was less than or equal to the ECV.

Table 2. Variation in susceptibility of *C. neoformans* to fluconazole over a 13-year period (1996-2008)[a].

Antifungal agent (ECV[µg/ml])	Region	Year	No. tested	MIC (µg/ml)[b]		% of MICs > ECV
				Range	Mode	
Fluconazole (8)	Asia-Pacific	2001-2004	294	0.25-32	4	4.1
		2005-2008	63	0.25-8	4	0.0
	Latin America	2001-2004	127	0.5-32	4	4.7
		2005-2008	63	0.25-16	4	1.6
	Europe	1996-2000	41	0.5-32	4	4.9
		2001-2004	63	1-16	4	3.2
		2005-2008	47	1-8	4	0.0
	North America	1996-2000	103	0.25-16	4	3.9
		2001-2004	140	0.25-128	4	2.9
		2005-2008	44	0.12-8	4	0.0
	All regions	1996-2000	144	0.25-32	4	4.2
		2001-2004	625	0.25-128	4	3.8
		2005-2008	217	0.12-16	4	0.5

a. Isolates submitted to and tested at the University of Iowa (Iowa City, Iowa, USA) as part of the ARTEMIS Antifungal Surveillance Program.
b. Results obtained after 72h incubation.

Table 3. Variation in susceptibility of *C. neoformans* to posaconazole and voriconazole over an 8-year period (2001-2008).

Antifungal agent (ECV[µg/ml])	Region	Year	No. tested	MIC (µg/ml)		% of MICs > ECV
				Range	Mode	
Posaconazole (0.25)	Asia-Pacific	2001-2004	294	0.015-2	0.12	5.1
		2005-2008	63	0.03-0.5	0.12	6.3
	Latin America	2001-2004	127	0.03-2	0.12	5.5
		2005-2008	63	0.03-0.5	0.12	1.6
	Europe	2001-2004	63	0.015-0.5	0.12	7.9
		2005-2008	47	0.03-0.25	0.12	0.0
	North America	2001-2004	139	0.015-2	0.12	5.0
		2005-2008	44	0.008-0.5	0.12	2.3
	All regions	2001-2004	623	0.015-2	0.12	5.5
		2005-2008	217	0.008-0.5	0.12	2.8
Voriconazole (0.12)	Asia-Pacific	2001-2004	292	0.008-0.5	0.06	2.7
		2005-2008	62	0.015-0.25	0.06	6.5
	Latin America	2001-2004	127	0.008-0.5	0.06	3.1
		2005-2008	63	0.008-0.25	0.06	1.6
	Europe	2001-2004	63	0.008-0.25	0.06	4.8
		2005-2008	47	0.015-0.12	0.06	0.0
	North America	2001-2004	139	0.007-1	0.06	3.6
		2005-2008	44	0.008-0.12	0.06	0.0
	All regions	2001-2004	621	0.008-1	0.06	3.2
		2005-2008	216	0.008-0.25	0.06	2.3

CONCLUSIONS

- We established the 72-h ECVs for fluconazole, posaconazole, and voriconazole, using an extensive geographically diverse clinical collection of *C. neoformans* isolates. Given the lack of clinical breakpoint concentrations for these agents against *C. neoformans,* the ECVs proposed herein may prove useful in detecting the emergence of potential resistance as these azoles are more often used.
- The very low frequency of strains with decreased susceptibility to fluconazole suggests that routine antifungal susceptibility testing of incident isolates may not necessary; however, it is still important to periodically monitor for the emergence of resistance.

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