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Effect of pH on *In Vitro* Susceptibility of *Candida glabrata* and *Candida albicans* to 11 Antifungal Agents and Implications for Clinical Use

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The treatment of vulvovaginal candidiasis (VVC) due to *Candida glabrata* is challenging, with limited therapeutic options. Unexplained disappointing clinical efficacy has been reported with systemic and topical azole antifungal agents in spite of *in vitro* susceptibility. Given that the vaginal pH of patients with VVC is unchanged at 4 to 4.5, we studied the effect of pH on the *in vitro* activity of 11 antifungal agents against 40 *C. glabrata* isolates and compared activity against 15 fluconazole-sensitive and 10 reduced-fluconazole-susceptibility *C. albicans* strains. *In vitro* susceptibility to flucytosine, fluconazole, voriconazole, posaconazole, itraconazole, ketoconazole, clotrimazole, miconazole, ciclopirox olamine, amphotericin B, and caspofungin was determined using the CLSI method for yeast susceptibility testing. Test media were buffered to pHs of 7, 6, 5, and 4. Under conditions of reduced pH, *C. glabrata* isolates remained susceptible to caspofungin and flucytosine; however, there was a dramatic increase in the MIC_{90} for amphotericin B and every azole drug tested. Although susceptible to other azole drugs tested at pH 7, *C. albicans* strains with reduced fluconazole susceptibility also demonstrated reduced susceptibility to amphotericin B and all azoles at pH 4. In contrast, fluconazole-sensitive *C. albicans* isolates remained susceptible at low pH to azoles, in keeping with clinical observations. In selecting agents for treatment of recurrent *C. glabrata* vaginitis, clinicians should recognize the limitations of *in vitro* susceptibility testing utilizing pH 7.0.

Vulvovaginal candidiasis (VVC) accounts for up to one third of all vaginitis cases presenting to gynecologists (1, 2, 3). VVC is most commonly caused by *Candida albicans* but can also be caused by non-*albicans Candida* species, with *Candida glabrata* being the most common (24, 26). Symptomatic *C. glabrata* vaginitis poses a significant problem for clinicians because effective treatment and eradication of *C. glabrata* from the vagina have proven difficult (23, 25, 26). The organism has variable intrinsic resistance to azole drugs (23, 25, 26). *C. glabrata* vaginitis has been moderately successfully treated with boric acid, but this is not curative in one third of patients (8, 23, 25, 26). Other therapies have been advocated, such as topical flucytosine, oral itraconazole, and nystatin suppositories (8, 18). Amphotericin B suppositories in patients with non-*albicans Candida* resistant to azoles were studied by Phillips and found to be promising; however, symptomatic *C. glabrata* vaginitis is often unresponsive to these regimens (11, 18). VVC is also occasionally caused by fluconazole-resistant *C. albicans*, posing a similar treatment dilemma in that susceptibility of these organisms to other azole and non-azole drugs is not clinically predictive (26).

Drug treatment of vaginal infections may be unique in that the normal pH of the vagina is 4 to 4.5, which remains unchanged during VVC (13). Previous studies have found that the test medium pH in *in vitro* susceptibility testing can alter the azole MIC for *Candida* species and that an acidic pH tends to increase the MICs of fluconazole for selected *Candida* species (16). However, it was concluded that more acidic conditions did not change the designation of the isolates from susceptible to resistant, neither were clinical implications evident. The purpose of this study was to determine whether a change in test medium pH had an effect on *in vitro* susceptibility of *C. glabrata* and both fluconazole-susceptible and reduced-susceptibility *C. albicans* to seven azole and four non-azole antifungal agents, in order to explain the frequent *in vivo* failure of these agents in women with vaginitis caused by *C. glabrata*.

MATERIALS AND METHODS

Vaginal isolates of *C. glabrata* and *C. albicans* were chosen from the Wayne State Vaginitis Clinic microbiology laboratory organism bank. The definition of fluconazole-susceptible *C. albicans* was the presence of an MIC of $\leq$2 μg/ml, and reduced susceptibility was defined as an MIC of $\geq$4 μg/ml (4). Vaginal isolates were randomly chosen from the years 2000 to 2010 and plated on CHROMagar to verify purity of culture. These plates were incubated for 48 h at 37°C in ambient air. Susceptibility testing was then performed using a broth microdilution method, according to CLSI document M27-A3 (2008) guidelines utilizing pH 7 (4). Antifungals and concentrations tested were flucytosine and fluconazole (at MIC ranges of 0.125 to 64 μg/ml), and voriconazole, posaconazole, itraconazole, ketoconazole, clotrimazole, miconazole, ciclopirox olamine, amphotericin B, and caspofungin (all with MIC ranges of 0.03 to 16 μg/ml). *C. albicans* isolates known to be fluconazole susceptible (MIC, $\leq$ 2 μg/ml) were not tested against itraconazole, ketoconazole, clotrimazole, and miconazole. A 0.1-ml yeast inoculum of 1.5 ($\pm$ 1.0) $\times 10^3$ cells/ml in RPMI 1640 medium was added to each microdilution well. The trays were then incubated at 35°C for 48 h in ambient air. The MICs were read as the lowest

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TABLE 1 MIC_{50} and MIC_{90} susceptibility results

Type (no.) of isolates	pH	Value (μg/ml)[a]														
		5FC			FLU			AMB			VORI			POSA		
		MIC_{90}	MIC_{50}	Range	MIC_{90}	MIC_{50}	Range	MIC_{90}	MIC_{50}	Range	MIC_{90}	MIC_{50}	Range	MIC_{90}	MIC_{50}	Range
Fluconazole-resistant *C. glabrata* (40)	7	0.125	0.125	0.125	32	8	2–>64	0.25	0.125	0.125–0.5	0.5	0.125	0.03–>16	0.5	0.125	0.03–>16
	6	0.125	0.125	0.125–2	>64	>64	2–>64	0.5	0.125	0.03–1	4	2	0.03–8	8	2	0.03–>16
	5	0.125	0.125	0.125–1	>64	>64	2.0–>64	1	0.25	0.25–2	>16	4	0.03–>16	>16	2	0.03–>16
	4	0.125	0.125	0.125–0.5	>64	>64	1–>64	4	4	2–8	>16	8	0.03–>16	>16	4	0.03–>16
Fluconazole-resistant *C. albicans* (10)	7	2	1	0.125–4	4	2	0.125–16	1	0.5	0.25–2	0.5	0.03	0.03–0.5	0.03	0.03	0.03
	6	2	0.5	0.125–2	32	8	0.5–32	2	2	2–4	1	0.25	0.03–16	0.06	0.03	0.03–16
	5	2	0.25	0.125–25	32	8	0.5–64	4	4	4	1	0.25	0.03–1	0.25	0.03	0.03–0.25
	4	>64	0.5	0.125–>64	>64	32	16–>64	16	16	8–16	>16	2	0.03–>16	16	0.03	0.03–16
Fluconazole-sensitive *C. albicans* (15)	7	1	0.125	0.125–2	0.25	0.125	0.125–0.25	0.5	0.25	0.25–0.5	0.03	0.03	0.03	0.03	0.03	0.03
	6	0.5	0.125	0.125–0.5	0.5	0.25	0.25–0.5	2	1	1–2	0.03	0.03	0.03	0.03	0.03	0.03
	5	0.25	0.125	0.125–2.5	0.5	0.5	0.25–1	4	4	4	0.03	0.03	0.03	0.03	0.03	0.03
	4	0.25	0.125	0.125–0.5	2	1	0.5–16	8	8	8–16	0.03	0.03	0.03	0.03	0.03	0.03

[a] 5FC, flucytosine; AMB, amphotericin B; CASPO, caspofungin; CPO, ciclopirox olamine; FLU, fluconazole; VORI, voriconazole; POSA, posaconazole; ITRA, itraconazole; KTZ, ketoconazole; CLO, clotrimazole; MIC, miconazole.

antifungal concentration with substantially lower turbidity (80% growth reduction) compared to growth in the antifungal-free growth well for all agents. Testing known ATCC strains of *Candida parapsilosis* and *Candida krusei* ensured quality control. Antifungal susceptibility testing was carried out for each isolate at pH 6, 5, and 4 using a MOPS (morpholinepropanesulfonic acid) (Sigma-Aldrich) buffer solution, and MIC ranges, medians, MIC_{50}s, and MIC_{90}s were compared.

RESULTS

A total of 40 vaginal strains of *C. glabrata* and 15 fluconazole-sensitive and 10 reduced-fluconazole-susceptibility *C. albicans* strains were studied, and MICs were recorded at pH levels 7, 6, 5, and 4 for each antifungal tested. Table 1 outlines MIC_{50} and MIC_{90} susceptibility results, including ranges of antifungal agents tested for each pH value for both *C. glabrata* and *C. albicans.*

C. glabrata. At pH 7, all *C. glabrata* isolates were susceptible to flucytosine, amphotericin B, caspofungin, and ciclopirox olamine. In contrast, a range of *in vitro* activity was present for the various azole agents. The MIC_{90} for fluconazole was 32 μg/ml (range 2 to >64 μg/ml) with considerably lower MICs for all other azoles tested. Notably low MICs were documented for posaconazole and voriconazole at 0.5 μg/ml. Itraconazole and ketoconazole were highly active at pH 7 and the topical agents clotrimazole and miconazole were similarly active.

With progressive reduction in pH, MIC_{90} values for 5-fluconazole and caspofungin were unchanged; however, an increase in MIC was evident for amphotericin B and to a lesser extent ciclopirox olamine. A dramatic increase in MIC_{90} was evident for all azoles tested to drug levels achievable in the vagina with systemic azole use, although pharmacologic data are not available. The trends observed for MIC_{90} were also reflected in MIC_{50} values.

C. albicans. At pH 7, fluconazole-susceptible strains of *C. albicans* were predictably susceptible to all antifungal agents tested. With a decrease in pH, a significant increase in MIC was evident only with amphotericin B and ciclopirox olamine. Azole activity at the lower pH was maintained in the fluconazole-susceptible isolates.

At pH 7, 10 vaginal isolates of fluconazole-reduced-susceptibility *C. albicans* were evaluated. The MIC range for fluconazole activity was 4 to >64 μg/ml, with MIC_{90} being 4 μg/ml. These isolates remained susceptible to all other azole drugs tested but demonstrated a moderately higher MIC to flucytosine (MIC, 2 μg/ml). In contrast, when a lower pH was tested, dramatic increases in MIC were seen for flucytosine, amphotericin B, fluconazole, posaconazole, voriconazole, itraconazole, and ketoconazole.

DISCUSSION

The results of this study reveal that different classes of antifungals and the two species of *Candida* studied *in vitro* behaved differently with decrease in pH. The results confirm the susceptibility of fluconazole-sensitive *C. albicans* isolates to all azoles and the variable resistance of *C. glabrata* to fluconazole, and they may also offer insight as to why some antifungal medications may not be as effective *in vivo* with a more acidic physiologic vaginal pH. Previous studies similarly found that the medium pH can alter azole MICs for *Candida* species, and specifically an acidic pH was reported to increase the MICs of fluconazole for selected *Candida* species (16). The clinical implications of this observation were not, however, recognized.

C. glabrata vaginal infection is by no means infrequent, but case numbers are insufficient to perform a randomized controlled trial in order to establish optimal treatment (23, 25, 26). The resistance of *C. glabrata* to fluconazole, at all pH levels, observed in the present study is consistent with numerous *in vitro* studies (19, 20) and reflects experience when treating vulvovaginal candidiasis (7, 20, 25) and bloodstream infections (12, 19). Posaconazole and voriconazole are frequently but not invariably active against fluconazole-resistant *C. glabrata*. The *Candida* surveillance study demonstrated that resistance to fluconazole was highly predictive for resistance to voriconazole (14). Sabatelli et al. studied 1,218 *C. glabrata* isolates and their resistance to different azoles and amphotericin B, concluding that isolates with elevated MICs to one azole were generally less susceptible to all azoles (22). An important new finding in the present study reveals that *C. glabrata* isolates resistant to fluconazole but susceptible to posaconazole and voriconazole at pH 7 are unlikely to be effectively treated *in vivo* given the dramatic increases in MIC to these drugs at pH 4 and 5.

The topical agents miconazole and clotrimazole, which achieve high local concentrations, are similarly likely to be ineffective. This conclusion is strongly supported by clinical experience (27). In contrast, flucytosine maintained activity at low pH, a finding that supports experience in successfully treating *C. glabrata*-affected woman with symptomatic vaginitis (25).

TABLE 1 (Continued)

Value (μg/ml)[a]																	
CASPO			CPO			ITRA			KTZ			CLO			MIC		
MIC_{90}	MIC_{50}	Range	MIC_{90}	MIC_{50}	Range	MIC_{90}	MIC_{50}	Range	MIC_{90}	MIC_{50}	Range	MIC_{90}	MIC_{50}	Range	MIC_{90}	MIC_{50}	Range
0.5	0.5	0.25–1	0.5	0.25	0.125–0.5	1	0.125	0.03–2	1	0.06	0.03–8	2	0.125	0.03–2	0.5	0.03	0.03–4
0.5	0.5	0.125–1	1	1	0.5–1	>16	>16	0.03–>64	4	2	0.03–4	4	2	0.03–8	1	0.25	0.03–2
0.5	0.5	0.03–1	1	1	0.5–2	>16	>16	0.03–>16	>16	8	0.03–>16	8	4	0.125–16	8	0.5	0.03–8
0.5	0.5	0.125–1	2	2	1–4	>16	1	0.25–>16	>16	>16	4–>16	>16	16	1–>16	16	2	0.06–16
0.125	0.06	0.03–0.125	0.5	0.25	0.03–0.5	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	1	0.125	0.03–1
0.06	0.03	0.03–0.0125	1	1	0.5–1	0.06	0.06	0.03–0.125	0.25	0.06	0.03–0.25	0.125	0.03	0.03–0.25	4	0.5	0.03–4
0.06	0.03	0.03–0.06	1	1	1–2	0.125	0.06	0.03–16	0.5	0.06	0.03–16	0.25	0.03	0.03–0.5	1	0.125	0.03–1
2	0.06	0.03–16	2	1	1–2	>16	0.03	0.03–>16	>16	0.5	0.03–>16	0.5	0.25	0.03–4	1	0.25	0.03–1
0.125	0.06	0.06–0.25	0.5	0.5	0.25–0.5												
0.125	0.06	0.03–0.06	1	0.5	0.5–1												
0.06	0.06	0.03–0.06	1	1	0.5–1												
0.25	0.06	0.03–0.06	2	1	1												

Topical amphotericin B has in small studies demonstrated effectiveness for treatment of non-*albicans Candida* vaginitis, but emerging resistance to this antifungal has been documented (11, 18). Topical amphotericin B has also been used in combination with other antifungals, such as flucytosine. If flucytosine is as effective as previously described and stable at a low pH, perhaps it is contributing much more than amphotericin B to successful treatment (25). It was found in the present study that for both *Candida* species, amphotericin B activity was profoundly affected by pH, with at least a 16-fold increase in MIC_{90} with decrease in pH.

Ciclopirox olamine is an agent applied topically and well known for its potency against dermatophytes, and it has been suggested as an antifungal for resistant VVC. It is a synthetic topical agent, widely used to treat onychomycosis, tinea pedis, pityriasis versicolor, and seborrheic dermatitis. Its use in treating vaginal candidiasis has also been studied, with limited success (9, 17, 21), and it has shown clinical promise against azole-resistant *Candida* species, including *C. glabrata.* It has demonstrated good topical and systemic tolerance in rats and rabbits when vaginal tissue was examined (5, 15) and has been studied in settings with a lower pH (9, 10). However, in this study, a 4-fold rise in MIC_{90} from 0.5 to 2 μg/ml with a decrease in pH was seen. One factor that limits the clinical application of these data is that the breakpoint of ciclopirox olamine is unknown and the clinical relevance of increased MICs is questionable.

Caspofungin is an echinocandin that has demonstrated activity against *Candida* species both *in vitro* and *in vivo* for systemic infections (6). None of the echinocandins are available as topical agents, and they have not yet been studied for vulvovaginal candidiasis or at decreased pH levels. The results of this study demonstrated stable MICs with a decrease in pH, with all *C. albicans* isolates having an MIC_{90} of less than 2 μg/ml, and continued activity against *C. glabrata* isolates at lower pH. Additional studies would need to be performed to evaluate echinocandin response *in vivo* as a topical compound.

This *in vitro* study demonstrates the potential limitations of conventional *in vitro* testing in predicting antifungal clinical success when faced with the challenge of treating recurrent vulvovaginal *C. glabrata* infections as well as fluconazole-refractory *C. albicans* vaginitis. Although the importance of medium pH in standardizing susceptibility testing is widely recognized in recommending routine testing at pH 7, the profound effect of pH on *C. glabrata* susceptibility has not been appreciated but is probably relevant only to patients with yeast vaginitis. The exact mechanism of pH-induced reduced susceptibility has not been established. In contrast, fluconazole-susceptible *C. albicans* strains responsible for the majority of vaginitis episodes are less vulnerable to the pH influence. Finally, *C. albicans* vaginal isolates already demonstrating reduced azole sensitivity at pH 7 are further compromised by lowering pH, resembling the effect seen with *C. glabrata*. This study also emphasizes the need for new alternate agents for treatment of *C. glabrata* vaginitis as well as to consider measuring *C. glabrata* drug susceptibility *in vitro* at pH 4 to 5 before recommending antimycotic therapy; however, validation studies are essential.

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