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Original Research

A Pilot Study Showing Fluconazole and Flucytosine Activities against *Candida glabrata* are Affected by Low pH: Implications for the Treatment of Recurrent Vulvovaginal Candidiasis

Ziauddin Khan[1], Suhail Ahmad[1,*], Mohammad Asadzadeh[1]

[1]Departments of Microbiology, Faculty of Medicine, Kuwait University Health Sciences Center, 46300 Jabriya, Kuwait
*Correspondence: suhail.ahmad@ku.edu.kw (Suhail Ahmad)
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Abstract

Background: *Candida albicans (C. albicans)* and *Candida glabrata (C. glabrata*) are mainly associated with vulvovaginal candidiasis (VVC). Management of VVC caused by *C. glabrata* is particularly challenging due to its inherent reduced susceptibility to fluconazole. In this prospective laboratory-based cohort study, we investigated the effect of pH on *in vitro* susceptibility of *Candida* spp. isolates to fluconazole and flucytosine. **Methods**: Vaginal isolates of *C. glabrata*, *C. albicans*, *Candida tropicalis (C. tropicalis)* and *Candida parapsilosis (C. parapsilosis)* were tested for susceptibility to fluconazole and flucytosine by Epsilometer test (ETEST) strips on Roswell Park Memorial Institute (RPMI) 1640 medium at pH 7.0 and pH 4.5. Minimum inhibitory concentrations (MICs) were read after 24 h at 35 °C. Results were interpreted according to the European Committee on Antimicrobial Susceptibility testing (EUCAST) guidelines. **Results**: Mean fluconazole MICs (µg/mL) at pH 4.5 were significantly higher than those at pH 7.0 for *C. glabrata* (82.55 ± 100.32 versus 14.96 ± 7.71, respectively, $p = 0.001$) and *C. albicans* (1.32 ± 7.98 versus 0.96 ± 1.35, respectively, $p = 0.017$) isolates. A similar effect was not observed with *C. tropicalis* and *C. parapsilosis* isolates. In contrast, mean MICs against flucytosine were reduced at pH 4.5 compared to pH 7.0 for all four *Candida* spp. isolates, with this reduction being statistically significant for *C. glabrata* and *C. parapsilosis* isolates. **Conclusions**: Our data show that the therapeutic efficacy of fluconazole against *C. glabrata* and *C. albicans* is reduced at lower (normal vaginal) pH values while the activity of flucytosine is enhanced. Therefore, flucytosine may serve as an effective alternative for the treatment of VVC and recurrent VVC caused by *C. glabrata* and other *Candida* spp.

Keywords: *Candida glabrata*; *Candida albicans*; fluconazole; flucytosine; *in vitro* susceptibility; pH effect

1. Introduction

The global incidence of mucocutaneous and invasive fungal infections have increased in the last two decades due to an expanding population of susceptible patients, and increasing use of antifungal prophylaxis [1–6]. *Candida* spp. play a crucial role in causing mucocutaneous and invasive fungal infections around the world [7–11]. The problem is further complicated by the emergence of drug-resistant and multidrug-resistant fungal pathogens in recent years [4,12–16]. For instance, the recently recognized multidrug-resistant *Candida auris (C. auris)* is now a major fungal pathogen in some countries [3]. *C. auris* has also caused outbreaks in healthcare facilities, which have been difficult to control [17,18]. While *Candida albicans (C. albicans)* is the most frequently isolated yeast species from clinical specimens and is considered the most pathogenic, non-*albicans Candida* species (NACS) currently constitute over 50% of all *Candida* infections [19–21]. *Candida glabrata (C. glabrata)* is also one of the most frequently isolated yeast species from bloodstream and other anatomical sites, especially from the critically ill patients and those over 65 years [7,19–21].

Vulvovaginal candidiasis (VVC) represents a worldwide problem, affecting approximately 75% of adult females of childbearing-age at least once in their lifetime [8,10,22,23]. The management of VVC becomes challenging when caused by some NACS, given their reduced susceptibility to fluconazole [8,10,22,23]. Furthermore, the ready availability of topical azoles and their widespread usage in certain countries for the treatment of VVC has resulted in the emergence of resistant strains [22,23]. As an example, *C. glabrata* isolates exhibit reduced susceptibility to fluconazole, and instances of resistance to echinocandins and amphotericin B have also been documented among clinical isolates [24–28]. Recurrent VVC (defined as at least three confirmed infections within a 12-month period) affects nearly 10% of women and presents a challenging treatment scenario [10,29]. In addition to oral fluconazole, various topical treatments such as probiotics, boric acid, honey, and other remedies have been employed, with variable success [30–33]. Studies have also shown that the vaginal environment in patients with VVC has a pH of 3.8 to 4.5, which negatively affects the activity of some antifungal drugs commonly used to treat VVC [34–36]. In this report, we have explored the effect of pH on *in vitro* susceptibility of vaginal isolates of *C. albicans*, *C. glabrata*,

Candida tropicalis (*C. tropicalis)* and *Candida parapsilosis* (*C. parapsilosis)* to fluconazole and flucytosine.

2. Materials and Methods

2.1 Research Object

A prospective laboratory-based cohort study was conducted by using 40 *Candida* spp. isolates selected from the stock culture collection maintained at Mycology Reference Laboratory, Department of Microbiology, Faculty of Medicine, Kuwait University. The isolates were cultured from vaginal specimens obtained from hospitalized patients, and verbal consent was obtained solely as part of routine patient care, for both the growth and antifungal susceptibility testing of fungal pathogens. A total of 15 vaginal isolates of *C. glabrata* and *C. albicans,* and 5 each of *C. tropicalis* and *C. parapsilosis* were used. *In vitro* susceptibility to fluconazole was determined by using Epsilometer test (ETEST) strips at pH 7.0 and pH 4.5, as described below (please see 2.3). For comparison, 5 vaginal isolates of *C. glabrata*, *C. albicans*, *C. tropicalis,* and *C. parapsilosis* were also tested against flucytosine at pH 7.0 and pH 4.5. The study was approved by the Health Sciences Center Ethics Committee (vide approval no. VDR/EC/3724), Kuwait University, and all the methods and investigations were carried out according to their guidelines. The need for informed consent was waived by the Health Sciences Center Ethics Committee, as there was no direct contact with the patients from whom *Candida* spp. isolates were obtained.

2.2 Phenotypic and Molecular Characterization of Clinical Isolates

The isolates were initially identified phenotypically by their characteristic colony color on CHROMagar Candida (CHROMagar, Paris, France) and subsequently by Vitek2 yeast identification system (BioMèrieux, Marcy-l'Étoile, France), performed according to the manufacturer's instructions and as described previously [37]. For genotypic identification, genomic DNA was extracted from each isolate by the boiling method using Chelex-100 (Sigma-Aldrich, St. Louis, MO, USA), as described previously [38]. Genotypic identification of *C. albicans C. glabrata*, *C. tropicalis* and *C. parapsilosis* isolates was achieved by polymerase chain reaction (PCR) or multiplex PCR amplification of rDNA by using species-specific primers, as described previously [38–41]. Briefly, DNA samples from *C. albicans* isolates were subjected to duplex PCR by using *C. albicans*-specific CALF, 5′-TGGTAAGGCGGGATCGCTT-3′ and CALR, 5′-GGTCAAAGTTTGAAGATATAC-3′ and *C. dubliniensis*-specific CDUF, 5′-AAACTTGTCACGAGATTATTTTT-3′ and CDUR, 5′-AAAGTTTGAAGAATAAAATGGC-3′ primers. PCR reactions were carried out by using the reaction and cyclic conditions and the amplicons were analyzed by agarose gel electrophoresis, as described previously [39]. To differentiate *C. albicans* from another closely related species, *Candida africana (C. africana)*, PCR amplification was also done with *HWP1* (CR-f, 5′-GCTACCACTTCAGAATCATCATC-3′ and CR-r, 5′-GCACCTTCAGTCGTAGAGACG-3′) primers, as described previously [42].

The DNA samples from *C. glabrata* isolates were amplified in multiplex PCR by using mCGLF, 5′-CGGTTGGTGGGTGTTCTGC-3′; mCNIF, 5′-GAGGAGTTTGTATCTTTCAACTT-3′; mCBRF, 5′-GGGACGGTAAGTCTCCCG-3′ and mCGCR, 5′-CACGGAATTCTGCAATTCACA-3′ primers, as described previously [41]. PCR amplification for *C. tropicalis* isolates was performed by using CTROPF (5′-TTTATTTACAGTCAAACTTGAT-3′) and CTROPR (5′-TTAAATTCTTTCAAACAAACC-3′) primers, as described previously [40]. The DNA samples from *C. parapsilosis* isolates were amplified in multiplex PCR by using mCPF, 5′-TTTGCTTTGGTAGGCCTTCTA-3′; mCOF, 5′-TAAGTCAACTGATTAACTAAT-3′; mCMF, 5′-AACTGCAATCCTTTTCTTTCTA-3′; mLEF, 5′-TACAGAATTTTGAGAATTGTG-3′ and mCPCR, 5′-AATATCTGCAATTCATATTACT-3′ primers, as described previously [38]. Species-specific amplification of DNA from the respective *Candida* spp. isolates has been established in previous studies [38–42]. The identity was further confirmed by PCR-sequencing of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) by using panfungal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and CTS1R (5′-TCTTTTCCTCCGCTTATTGATATGC-3′), as described previously [43].

2.3 Antifungal Susceptibility Testing by ETEST

The ETEST strips of fluconazole and flucytosine were procured from BioMèrieux (Marcy-l'Étoile, France). The ETEST was carried out by using Roswell Park Memorial Institute (RPMI) 1640 agar medium supplemented with 2% glucose at pH 7.0 and pH 4.5 using morpholinepropane sulphonic acid (MOPS) buffer, as described previously [44]. Briefly, the turbidity of the yeast cell suspension was adjusted to 0.5 McFarland standard. Using sterile cotton swabs, the yeast suspension was evenly spread over the entire surface of the RPMI agar medium plate. The ETEST strips were placed on the surface of the medium and the plates were incubated at 35 °C. The minimum inhibitory concentration (MIC) values were read after 24 h of incubation at the lowest drug concentrations at which the zone of inhibition intersected the strip scale. The European Committee on Antimicrobial Susceptibility testing (EUCAST) MIC breakpoints for fluconazole were as follows: $\leq$2, 4 and $\geq$8 µg/mL corresponding to susceptible, susceptible dose-dependent and resistant, respectively, for *C. albicans*, *C. tropicalis,* and *C. parapsilosis,* and $\leq$32 and $\geq$64 µg/mL corresponding to susceptible dose-dependent and resistant, respectively, for *C. glabrata*. Given the absence of estab-

lished clinical breakpoints or epidemiological cutoff values (ECVs) for flucytosine, we applied an ECV of 1 µg/mL for flucytosine for all four *Candida* spp. isolates, consistent with the approach of other recent studies [45,46].

2.4 Statistical Analyses

Susceptibility data (MIC values) were analyzed with IBM SPSS Statistics software (version 22, IBM Corp., Armonk, NY, USA). The mean MIC value for each antifungal drug was calculated. Differences in antifungal susceptibility patterns among *Candida* spp. at the two pH values were calculated by using non-parametric Wilcoxon signed-rank test. A p value of $\leq$0.05 was considered as statistically significant.

3. Results

A total of 40 *Candida* spp. isolates cultured from vaginal specimens comprising *C. albicans* (n = 15), *C. glabrata* sensu stricto (n = 15), *C. tropicalis* (n = 5) and *C. parapsilosis* sensu stricto (n = 5) were tested. All isolates were correctly identified to the species level by their colony characteristics on CHROMagar Candida. Subsequently, their assimilation profiles were confirmed by Vitek2 yeast identification system. All *C. albicans* isolates (n = 15) yielded an amplicon of ~120 bp in duplex PCR, which facilitated the differentiation from *C. dubliniensis*. The presence of *C. africana* among *C. albicans* isolates was ruled out by the PCR assay targeting the *HWP1* gene. All 15 isolates yielded an amplicon of ~941 bp which is specific for *C. albicans* [42]. All *C. glabrata* isolates (n = 15) yielded an amplicon of ~360 bp in multiplex PCR which is specific for *C. glabrata* sensu stricto strains. Similarly, PCR amplicons of ~260 bp and ~170 bp were successfully obtained from *C. tropicalis* (n = 5) and *C. parapsilosis* (n = 5) isolates, respectively, consistent with the expected results. The DNA sequences of the ITS region of rDNA from the isolates exhibited <1% difference with the sequence from the reference strains of the corresponding species, as expected.

The mean fluconazole MICs of 14.96 $\pm$ 7.71 µg/mL (geometric mean $\pm$ standard deviation (SD)) were obtained at pH 7.0 for 15 *C. glabrata* isolates. When the same 15 isolates were tested at pH 4.5, the mean fluconazole MICs increased significantly (82.55 $\pm$ 100.32 µg/mL, p = 0.001). The increase in MICs led to change in the classification of some *C. glabrata* isolates from 'susceptible dose-dependent' to 'resistant' to fluconazole (Table 1). Similarly, the mean fluconazole MICs for *C. albicans* isolates (n = 15) were also higher at pH 4.5 (1.32 $\pm$ 7.98 µg/mL) compared to the values at pH 7.0 (0.96 $\pm$ 1.35 µg/mL), and this disparity was also statistically significant (p = 0.017), as shown in Table 1. However, the mean fluconazole MICs for *C. tropicalis* (n = 5) and *C. parapsilosis* (n = 5) showed only slight increases when tested at pH 4.5 compared to pH 7.0, and these differences were not found to be statistically significant (Table 1). Unlike fluconazole MIC results, the mean flucytosine MICs for *C. glabrata*, *C. albicans*, *C. tropicalis* and *C. parapsilosis* isolates were lower at pH 4.5 compared to the MIC values at pH 7.0. Notably, the differences were statistically significant for *C. glabrata* (p = 0.042) and *C. parapsilosis* (p = 0.041), though not for *C. albicans* (p = 0.066) and *C. tropicalis* (p = 0.068) isolates (Table 2).

4. Discussion

The VVC represents a serious public health issue globally which causes considerable morbidity. Approximately 75% women of childbearing age experience at least one episode of VVC in their life-time, with nearly 10% experiencing recurrent VVC [8,10,22,23]. While recurrent VVC may not be a life-threatening condition, it affects the quality of life, causing both physical discomfort and psychological symptoms in the affected individuals. Several common antifungal drugs, including both conventional and newer drugs, as well as various topical remedies, have been used with varying degrees of success in the treatment of VVC [31–33,36,47,48]. The causative agents of VVC mainly include *C. albicans* and *C. glabrata* [8,22]. *C. africana*, a rare yeast species mostly associated with VVC, is genotypically very closely related to *C. albicans* and so, it is often misidentified as *C. albicans* by routine molecular testing [8,42,49]. Despite previous isolations of *C. africana* in Iran and Turkey within the Middle East [49,50], extensive attempts to identify it among clinical *C. albicans* isolates in Kuwait have not been successful (Z. Khan and S. Ahmad, Unpublished data). Moreover, *C. africana* was also not detected among the 15 *C. albicans* isolates examined in this study.

Considering the challenges associated with the successful treatment of VVC, likely due to the acidic vaginal environment, the effect of pH on *in vitro* susceptibility of vaginal isolates of *C. albicans* and *C. glabrata* to fluconazole and flucytosine was investigated. Additionally, two other less commonly encountered *Candida* spp. from vaginal specimens, *C. tropicalis* and *C. parapsilopsis,* were also included.

Consistent with an earlier study [34], our *in vitro* susceptibility data also showed significantly higher mean fluconazole MICs at normal vaginal pH (4.0–4.5) compared to the values obtained at normal physiological pH (pH 7.0) for both *C. glabrata* and *C. albicans* isolates. Our findings are thus in agreement with earlier studies indicating reduced therapeutic efficacy of fluconazole for VVC caused by the two most common agents, i.e., *C. glabrata* and *C. albicans* [22,33–35]. Although fluconazole MICs were also increased at lower pH for *C. tropicalis* and *C. parapsilopsis*, the differences were not statistically significant, most likely due to small number of isolates tested. Similar observations have also been made in another recent study. Spitzer *et al.* [35], in a study using first yeast isolates collected from 217 patients with VVC, showed that *in vitro* potency of four (ter-

Table 1. Effect of pH on susceptibility testing of vaginal *Candida* spp. isolates to fluconazole.

Candida species	No. of isolates	Test pH	MICs* (µg/mL) (Mean ± SD)	*p*-value
C. glabrata	15	7	14.96 ± 7.71	
		4.5	82.55 ± 100.32**	0.001
C. albicans	15	7	0.96 ± 1.35	
		4.5	1.32 ± 7.98	0.017
C. tropicalis	5	7	0.48 ± 0.22	
		4.5	0.60 ± 0.17	0.157
C. parapsilosis	5	7	0.63 ± 0.45	
		4.5	1.27 ± 0.27	0.066

*Geometric mean (GM) ± standard deviation (SD) of minimum inhibitory concentrations (MICs) (µg/mL). Three *C. glabrata* isolates yielded MICs of ≥256 µg/mL. **The GM calculated at MIC value of 256 µg/mL. *C*, *Candida*.

Table 2. Effect of pH on susceptibility testing of vaginal *Candida* spp. isolates to flucytosine.

Candida species	No. of isolates	Test pH	MICs* (µg/mL) (Mean ± SD)	*p*-value
C. glabrata	5	7	0.014 ± 0.004	
		4.5	0.008 ± 0.002	0.042
C. albicans	5	7	0.044 ± 0.129	
		4.5	0.015 ± 0.023	0.066
C. tropicalis	5	7	0.021 ± 0.013	
		4.5	0.01 ± 0.007	0.068
C. parapsilosis	5	7	0.011 ± 0.006	
		4.5	0.004 ± 0.002	0.041

*Geometric mean ± standard deviation (SD) of minimum inhibitory concentrations (MICs) (µg/mL).

conazole, clotrimazole, miconazole and fluconazole) azole drugs was reduced when these antifungals were tested at pH 4.0 compared to their activity at pH 7.0. Furthermore, the reduced potency of these drugs was more pronounced for *C. glabrata* than for *C. albicans* isolates [35]. For terconazole, mean MIC values increased nearly 40-fold (from 0.17 µg/mL at pH 7.0, to 6.17 µg/mL at pH 4.0) for *C. albicans* isolates. However, the increase in mean MICs for *C. glabrata* was nearly 65-fold as all isolates yielded an MIC of ≤1 µg/mL at pH 7.0, which increased to >64 µg/mL at pH 4.0 for nearly all the isolates [35].

Our findings also show that mean flucytosine MICs for *C. glabrata* were significantly lower at pH 4.5 (p = 0.042), and although not statistically significant (p = 0.066) for *C. albicans,* they were lower compared to the mean MICs at pH 7.0. These results suggest that topical flucytosine can be used as an effective therapeutic option for VVC caused by *C. glabrata* as well as for fluconazole-resistant and -susceptible strains of *C. albicans*. The mean flucytosine MICs at pH 4.5 were also lower for *C. tropicalis* and significantly lower for *C. parapsilosis* compared to the mean MICs at pH 7.0 for these two species. Furthermore, two previous studies have also reported favorable outcome with therapy regimens, including flucytosine in patients with VVC. Sobel *et al.* [30] reported 90% success following two weeks of intra-vaginal application of 17% flucytosine cream in patients with VVC who failed boric acid therapy. Similarly, White *et al*., [48] used lubricating jelly base containing flucytosine and amphotericin B for treatment of refractory vaginal candidiasis due to highly azole-resistant *C. glabrata*. These studies along with our preliminary results, indicate the importance of confirming these observations by testing a larger and more diverse collection of vaginal isolates from various dominant genotypes of *C. glabrata*, *C. albicans*, *C. africana*, *C. tropicalis* and *C. parapsilosis* for better management of VVC [30,48].

Clinical trials have recently shown that Ibrexafungerp exhibits an efficacy similar to fluconazole in the treatment of VVC [51]. In a recent study, susceptibility testing of fluconazole-resistant (n = 52) and susceptible (n = 30) *C. albicans* and *C. glabrata* (n = 25) isolates from women with VVC against the new antifungal agent, Ibrexafungerp, also showed that the MIC values were not affected for *C. albicans* and were only slightly elevated for *C. glabrata* isolates when tested at pH 4.0 compared to pH 7.0 [36]. These findings suggest that antifungal drugs with excellent activity at pH 4.0 to pH 4.5 will have a better therapeutic outcome for patients with VVC and recurrent VVC. Taken to-

gether, these studies and our data, suggest that flucytosine either alone or in combination with newer drugs like Ibrexafungerp, could be considered as an attractive alternative for the treatment of patients with VVC or recurrent VVC [30,36,48].

Our study has few limitations. (1) Only a small number of vaginal isolates of *C. glabrata*, *C. albicans*, *C. tropicalis* and *C. parapsilosis* were tested for their *in vitro* susceptibility to fluconazole and flucytosine at the two pH values. The study needs to be repeated with a larger number of *Candida* spp. isolates recovered from patients with VVC. (2) The antifungal susceptibility testing was performed by ETEST and not by the reference broth microdilution method.

5. Conclusions

Consistent with other published reports, our data also showed that the antifungal activity of fluconazole is reduced at pH 4.5 compared to its activity at pH 7.0 for *Candida* spp. that cause VVC, particularly for *C. glabrata* isolates. In contrast, flucytosine MICs against *C. glabrata* and *C. albicans* isolates were lower at normal vaginal pH value (pH 4.5) compared to the MICs at pH 7.0. These observations, along with recent reports on the excellent activity of the newer antifungal drug Ibrexafungerp at reduced pH, suggest that topical flucytosine, either used alone or in combination with Ibrexafungerp, may present itself as an attractive alternative for patients with VVC or recurrent VVC.

Availability of Data and Materials

All the data are available in the manuscript file. Other details will be provided by the corresponding author upon reasonable request.

Author Contributions

ZK: conceived the idea, project development, data collection and analysis, manuscript writing; MA, SA: data collection and analysis, manuscript writing. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work. All authors contributed to revisions of the manuscript and approved the final manuscript.

Ethics Approval and Consent to Participate

The study was approved by the Health Sciences Center Ethics Committee (vide approval no. VDR/EC/3724), Kuwait University and all the methods and investigations were carried out according to their guidelines. The need for informed consent was waived by the Health Sciences Center Ethics Committee as no direct contact was made with the patients yielding *Candida* spp. isolates.

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Conflict of Interest

The authors declare no conflict of interest.

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