

# Susceptibility to Fluconazole and Ketoconazole of *Candida* spp. Isolated from Primary and Episodic Vulvovaginites by E-Test (São Paulo, SP, Brazil)

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## Abstract

**Purpose:** To evaluate the profile of *in vitro* susceptibility of yeasts isolated from cases of primary and episodic vulvovaginitis to two antifungal agents. **Methods:** 40 *Candida* isolates from episodes of vulvovaginal candidiasis were identified by classic methodologies. The susceptibility testing of the *in vitro* fluconazole and ketoconazole activity against the isolates was accessed by E-test. **Results:** *C. albicans* was the most common species identified in 70% of the occurrences followed by *C. glabrata* (20%), *C. tropicalis* (7.5%), and *C. guilliermondii* (2.5%). In the susceptibility profile to antifungal agents, 12.5% and 16.7% of the isolates obtained from primary and episodic vulvovaginal candidiasis were resistant to fluconazole, respectively. To ketoconazole, we found that 6.25% and 12.5% of the isolates respectively from primary vulvovaginal candidiasis (PVVC) and episodic vulvovaginal candidiasis (EVVC) had high MIC values. **Conclusions:** E-test is a reliable method for the susceptibility testing of *Candida* spp. due to its simplicity, reproducibility, and lack of specialized equipment. Resistant strains and non-*albicans* species were verified more in cases of EVVC than in PVVC. Clinical and mycological cure of patients with episodic vulvovaginal candidiasis or complicated cases occurred after prolonged treatment and sometimes with multiple antifungals use.

## Keywords

Antifungal Susceptibility, *Candida*, Candidiasis Vulvovaginal, E-Test, Non-*Albicans* Species

## 1. Introduction

Vulvovaginal candidiasis (VVC) is classified as primary or non-complicated when manifested by sporadic or infrequent episodes with mild or moderate symptoms in healthy and non-pregnant woman, while complicated episodic and recurrent cases of *Candida* vulvovaginitis have more severe symptoms and signs with high frequency of resistant yeast species [1] [2]. Changes of pH, vaginal microbiota, and the endogenous host factors may trigger the disease [1]-[4]. *Candida albicans* has been the most common causal agent and non-*albicans* species have been increasingly identified [4]-[7]. The overall increase of resistant microorganisms and the consequent occurrence of refractory cases for treatments commonly used have been quicken for the interest in researches to development of new antimicrobial drugs, therapeutic schemes and susceptibility tests [5] [8] [9].

Azole substances have been used as the first-line of antifungal agents for treatment of VVC. However, the susceptibility obtained *in vitro* is not predictive of therapeutic success [10] [11]. On the other hand, the resistance could be a factor involved in inefficacy of antifungal drug therapy mainly in refractory cases [5] [12] [13].

E-test has been considered as an alternative to the reference broth microdilution method (CLSI) for its practicability and reproducibility [14]-[16]. It is easy, fast, and can be used by the gynecologist for increased reliability of prescription medication, especially in more complicated cases [15]-[17]. Comparison between the E-Test and microdilution showed high reliable correlation to fluconazole, voriconazole and caspofungin [18].

Due to the importance of vulvovaginal candidiasis at gynecological practice and the few Brazilian works on the fungal susceptibility testing of the strains isolated from VVC, the aim of this investigation was to evaluate the *in vitro* susceptibility to fluconazole and ketoconazole of *Candida* spp. isolated from primary and episodic cases assessed by E-Test in São Paulo (Brazil).

## 2. Material and Methods

In a prospective study conducted during two years, evaluated in private and public gynecological services in the greater São Paulo city (São Paulo State, Brazil), 562 women between the ages 18 and 65 years were followed up, and 168 of them with clinical suspicion of VVC were carefully investigated. Itching, discharge, erythema, dysuria, vulvar burning, and pain were the signs and symptoms analyzed.

According to the intensity, an arbitrary punctuation was attributed for each manifestation following the score: zero to three—was not considered; four to six-mild; seven to 13-moderate; 14 to 18-severe. The criteria of inclusion in this investigation were: patients with age range from 18 to 65 years, with positive cultures for *Candida* spp., intensity of signs and symptoms equal to greater than four, not pregnant, without any immunocomprising conditions. After the exclusion of women with diabetes mellitus, immuno-deficiency as HIV positive, in use of vaginal douches, spermicides, or intra-uterine device (IUD), and on steroid, hormone or antibiotic therapy, 40 patients with

clinical and laboratory diagnosis of VVC were selected; 16 patients with primary vulvovaginal candidiasis (PVVC) as group A and 24 with episodic vulvovaginal candidiasis (EVVC) as group B. Topical or systemic azole, or the combination of both was used for treatment and the patient evolution was carefully monitored.

The vaginal secretions were collected with non-lubricated speculum from the ectocervix and posterior vaginal fornix, using moistened swab in sterile saline solution. This procedure was performed after science and signing of agreement each patient. The study was approved by the Ethics Committee of the Federal University of São Paulo under n° 1719/05 and conducted according to the Helsinki Declaration revised in 2008.

One part of the samples was submitted to direct microscopy with KOH 20% more ink Parker (three to one) and smears were stained by Gram method. The other part was seeded on Sabouraud dextrose agar media supplemented with chloramphenicol (100 mg/ml) and CHROMagar *Candida* for screening of presumptive species. The yeast identification was performed according to morphological, biochemical and physiological characterization of the isolated species. *Candida albicans* identification was confirmed by the presence of chlamidoconidia in cornmeal agar tween 80, production germ tube at 37°C in serum and growth in hypertonic media at 42°C.

The susceptibility test of the isolates was performed by kit E-Test (AB Biodisk, Solna, Sweden) and the protocol followed the manufacturer's instructions. The assay medium RPMI 1640 agar supplemented with MOPS, L-glutamine, and glucose (HiMedia Laboratories, Bombay, India) was prepared and distributed in 90 mm-diameter Petri-dishes. *Candida* isolates were previously cultivated for 24 h on Sabouraud dextrose agar and inoculated on tubes with 3 mL of sterile saline, turbidity of 0.5 McFarland scale and then seed with sterile swabs on the surface of the medium. After 15 min of drying at room temperature, the E-Test strings of fluconazole and ketoconazole were placed on the agar surface and the plates were incubated at 37°C. The readings for the minimal inhibitory concentration (MIC) for the antifungal drugs were performed after 24 and 48 h of incubation. Interpretation of the results for was based on the values of MIC recommended by documents CLSI M27 A2, CLSI M27 A3 and CLSI M27 S4 [19]-[21]. For the ketoconazole there are not established breakpoints. The reference strain *Candida parapsilosis* ATCC 22019 was included for quality control. All experiments were performed in triplicate.

The Chi-Square tests were used in order to correlate the intensity of clinical forms of PVVC and EVVC and the *in vitro* susceptibility profile to fluconazole and ketoconazole.

### 3. Results

In this investigation, we analyzed 40 strains of *Candida* species, 26 from PVVC and 24 from EVVC. *Candida albicans* (70%) was the prevalent species, followed by *C. glabrata* (20%), *C. tropicalis* (7.5%) and *C. guilliermondii* (1.5%). The relationship of *Candida* species isolated from patients with PVVC and EVVC are described in **Table 1**. *Candida glabrata* was the prevalent non-*albicans* species isolated mainly in EVVC.

The intensity of signs and symptoms according to the species of *Candida* are represented in **Table 2**. The major number of patients (72.5%) presented a moderate degree of

**Table 1.** *Candida* species isolated from 40 cases of primary and episodic VVC.

Species	PVVC n (%)	EVVC n (%)	Total n (%)
<i>C. albicans</i>	13 (32.25)	15 (37.5)	28 (70)
<i>C. glabrata</i>	1 (2.5)	7 (17.5)	8 (20)
<i>C. tropicalis</i>	2 (5)	1 (2.5)	3 (7.5)
<i>C. guilliermondii</i>	-	1 (2.5)	1 (2.5)

VVC = vulvovaginal candidiasis, PVVC = primary VVC, EVVC = episodic VVC.

**Table 2.** Signs and symptoms insensitivity of VVC according to identified species.

Species	Mild n (%)	Moderate n (%)	Severe n (%)	Total n (%)
<i>C. albicans</i>	8 (20)	20 (50)	-	28 (70)
<i>C. glabrata</i>	2 (5)	6 (15)	-	8 (20)
<i>C. tropicalis</i>	-	2 (5)	1 (2.5)	3 (7.5)
<i>C. guilliermondii</i>	-	1 (2.5)	-	1 (2.5)
Total	10 (25)	29 (72.5)	1 (2.5)	40 (100)

VVC. Mild symptoms and signs were observed in 10 women (25%) and a severe presentation occurred only in one patient (2.5%). We do not observed correlation between symptoms and signs, manifestations type (PVVC and EVVC) and identified species. The most common age ranging was verified in patients from 18 to 32 years old.

In PVVC, only two isolates (one *C. albicans* and one *C. glabrata*, 12.5%) of 16 isolates showed resistance to fluconazole (**Table 3**). In EVVC, five isolates (one *C. albicans* and 4 *C. glabrata*) showed high MICs for fluconazole, and 4 isolates were SDD (3 *C. glabrata* and 1 *C. guilliermondii*).

In relation to ketoconazole, four isolates of *C. glabrata* (one from PVVC and three from EVVC, respectively 6.25% and 12.5%), two *C. albicans* strains (from PVVC and EVVC), two *C. tropicalis* strains (from PVVC), and the single-isolate *C. guilliermondii* showed high MICs > 16 µg/ml. Five isolates, *C. glabrata* (4) and *C. albicans* (1) have proved to be both resistant to fluconazole and high MICs values to ketoconazole. Trailing phenomenon was recorded in 25% of isolates.

*In vitro* susceptibility profile of the isolates to antifungal fluconazole and ketoconazole showed no significant clinical correlation with PVVC or EVVC and signs and symptoms in VVC.

In PVVC (group A) there was clinical and mycological cure after the first treatment with fluconazole 150 mg orally once weekly for two weeks. In this group, two samples (12.5%) were resistant to antifungal, one *C. albicans* isolate (6.25%) and one *C. glabrata* isolate (6.25%). The isolates obtained from the other 14 patients, *C. albicans* (12) and *C. tropicalis* (2) were susceptibility to fluconazole.

In EVVC (group B), 16 patients achieved clinical and mycological cures after the second treatment with fluconazole 150 mg orally once weekly for 3 weeks. In six patients, clinical and mycological cure occurred after the third treatment with ketoconazole orally at 400 mg daily for 14 days. And in the other two patients in this group, healing

**Table 3.** *In vitro* susceptibility profile to fluconazole of *Candida* species isolated from 40 cases of primary (P) and episodic (E) VVC, assessed by E-Test.

Species	S P/E	SDD P/E	R P/E	Total
<i>C. albicans</i>	12/15	0/0	1/0	28
<i>C. glabrata</i>	0/0	0/3	1/4	8
<i>C. tropicalis</i>	2/1	0/0	0/0	3
<i>C. guilliermondii</i>	0/0	0/1	0/0	1
Total	14/16	0/4	2/4	40

VVC = vulvovaginal candidiasis, S = susceptible, SDD = susceptible-dose dependent, R = resistant.

was given after the fourth treatment with fluconazole 150 mg orally weekly, for 4 weeks up to 6 weeks. We emphasize the occurrence of a higher strength and high MIC observed for *C. glabrata*, including a little therapeutic response especially in EVVC.

#### 4. Discussion

Vulvovaginal candidiasis is an insidious infection that affects a large proportion of female patients, most frequently in the reproductive age [1]-[4]. The distinction between bacterial and fungal vaginitis in relation to symptoms and etiological agent contributes to the therapeutic success [22]. Although *C. albicans* is the most frequently isolated species, other species have been found as infectious agents such as *C. glabrata*, *C. tropicalis* and *C. guilliermondii* [5] [6] [10] [23]. In our study, *C. albicans* and *C. glabrata* were the two most prevalent species. The *C. glabrata* strains were more resistant to antifungal agents commonly used in clinical practice. The resistant species were also frequently involved in relapse after treatment. The relapses were associated with prolongation of time action and consequently higher dosage of the drugs, and sometimes with the use of more than one antifungal during the treatment for VVC.

A recent study using the microdilution methods and disk diffusion resistance recorded *Candida* spp. emphasizes the need for standardization of methods that allow a better correlation between the resistance patterns and clinical manifestations [24]. Fluconazole resistance in species of *C. albicans* has not been shown as very common [10] [23] [25]-[29] on average less than 10% of the VVC isolated, while in *C. glabrata* resistant or susceptible-dose dependent occurring frequently around or more than 50% of the strains [5] [30]-[34]. In this investigation, 3.5% and 62.5% of the isolates of *C. albicans* and *C. glabrata*, respectively, proved to be resistant to fluconazole. Most yeast isolates from patients with EVVC, similar to those of the PVVC, in our research, were susceptible to fluconazole and ketoconazole, except *C. glabrata*. A study conducted for 8 years in China, with 2.204 isolates of *Candida* spp revealed prevalence of *C. albicans*, but an increase of non-*albicans* species such as *C. glabrata* over the years and antifungal resistance [35]. Unlike our study, in other Chinese research, high rate of resistant isolates for the ketoconazole was detected in 186 *Candida* strains from patients with VVC [36], observing resistance rate 27.7% in *C. albicans* strains and 56.2% in non-*albicans*

species.

Cross-resistance between azole antifungals such as fluconazole and ketoconazole is not uncommon fact [28] [32] [36] [37]. In our study, five (12.5%) isolates were resistant to fluconazole also showed great MICs values to ketoconazole. The selective pressure generated by the previous use of these two antifungal agents in cases of relapse is possibly correlated to the emergence of resistant strains isolated [31] [32] [38]. Fluconazole is inducing agent to selection of *C. albicans* with homozygous profile to *MTL* gene fast mode and high-frequency [39]. Treatment failure of CVV, in case of relapse or refractory, may also be associated with decreased susceptibility to the antifungal agent [28] [40]. Our investigation found susceptibility dose dependent (SDD) of *C. guilliermondii* to fluconazole and ketoconazole. This isolate was from second episode. This is consistent with other studies that shown *C. guilliermondii* as an emerging yeast with reduced susceptibility to antifungal agents [6] [23] [24] [41] [42].

Finally, we denote that susceptibility testing to antifungal agents of the isolated yeast of VVC, especially those of clinical significance, is important for detecting resistant microorganisms and may contribute to medical management and consequent therapeutic success, especially in episodic or complicated fungal vulvovaginitis. The intrinsic resistance of many strains and the antifungal drugs used for a long term or inappropriate treatment can leave to resistant-yeast selection among the fungal species [33]. On the other hand, antifungals used for a long period can leave susceptible or susceptible dose-dependent microorganisms to become more resistant to multiple drugs [11]. The use of E-test method is interesting as a screening method due to its practicality and a presumptive result of degree of susceptibility. Isolates that showed elevated MIC for the two tested antifungal agents were mainly *C. glabrata*, and much lower percentages of *C. albicans*, the two species most commonly found in this study. Further investigations with more VVC cases are necessary in order to analyze other factors related to the host and to fungal agents that will add to knowledge of the pathogenesis and maintenance of CVV.

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