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SPECIES DISTRIBUTION AND ANTIFUNGAL SUSCEPTIBILITY OF YEASTS ISOLATED FROM VAGINAL MUCOSA

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ABSTRACT

Aims: Vulvovaginal candidiasis is one of the most common complaints in the medical clinic. In recent years, due to the increasing frequency of non-*albicans* species, the number of cases of therapeutic failure has increased considerably, generating the need for research to learn the profile of yeasts isolated in vulvovaginal candidiasis. The aims of this study were to collect samples of vaginal secretion, verify the presence of yeast, identify the species of yeast, and verify their in vitro susceptibility profile against six antifungal agents – amphotericin B, nystatin, fluconazole, itraconazole, ketoconazole and voriconazole. *Materials and Methods:* Vaginal secretion was collected from 130 patients with symptoms characteristic of vulvovaginal candidiasis (VVC). For amphotericin B, fluconazole, itraconazole, ketoconazole and voriconazole, the in-vitro tests were carried out using the commercial Etest susceptibility testing kit; for nystatin the disk diffusion method was used. *Results:* The yeasts isolated were: *Candida albicans* (90%), *C. glabrata* (5%), *C. parapsilosis* (3%) and *C. tropicalis* (2%). By the CLSI method, all the isolates were susceptible to fluconazole, ketoconazole, nystatin and voriconazole. Tests showed that 98.8% of the isolates were susceptible to amphotericin B and 97.6% to itraconazole. *Conclusion:* Although a high number of resistant strains were not found, these studies may help guide physicians to the most convenient therapeutic orientation, conducting treatment specific to the identified yeast species.

KEY WORDS: *Candida* spp.; Vulvovaginal candidiasis; drug susceptibility.

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RESUMO

Distribuição das espécies e susceptibilidade aos antifúngicos de leveduras isoladas da mucosa vaginal.

Candidíase vulvovaginal é uma das queixas mais comuns na clínica médica. Nos últimos anos, devido ao aumento da frequência das espécies não albicans, o número de casos em que há falha terapêutica também aumentou, gerando a necessidade de pesquisas para conhecer o perfil das leveduras isoladas nos casos de candidíase vulvovaginal. Os objetivos deste estudo foram coletar amostras de secreção vaginal, verificar a presença de leveduras, identificar as espécies mais frequentes, e verificar o seu perfil *in vitro* de suscetibilidade frente a seis agentes antifúngicos – anfotericina B, nistatina, fluconazol, itraconazol, cetoconazol e voriconazol. *Material e Métodos*: A secreção vaginal foi coletada de 130 pacientes com sintomas característicos da candidíase vulvovaginal (CVV). Para anfotericina B, fluconazol, itraconazol, cetoconazol e voriconazol, os testes *in vitro* foram realizados utilizando o kit de sensibilidade comercial Etest, para nistatina, o método utilizado foi utilizado de difusão em disco. *Resultados*: As leveduras isoladas foram: *C. albicans* (90%), *C. glabrata* (5%), *C. parapsilosis* (3%) e *C. tropicalis* (2%). Pelo método CLSI, todas as amostras foram sensíveis ao fluconazol, cetoconazol, nistatina e voriconazol. Os testes mostraram que 98,8% dos isolados foram sensíveis à anfotericina B e 97,6% para itraconazol. *Conclusão*: Embora, não tenha sido encontrado um grande número de isolados resistentes, este estudo pode auxiliar o médico, na escolha da orientação terapêutica mais conveniente visando à realização de tratamento para as espécies de leveduras identificadas.

DESCRITORES: *Candida* spp; candidíase vulvovaginal; sensibilidade às drogas.

INTRODUCTION

Vulvovaginitis is a very common complaint in medical practice. About 75% of all women will have vulvovaginal candidiasis at some point in their lives and approximately 50% will have a second episode of the illness. Most cases of vulvovaginitis are easily treatable. However, approximately 5% of these patients will have recurrent vulvovaginitis showing frequent and hard-to-treat episodes (14, 23, 25).

Candida species are the second most common cause of vulvovaginitis worldwide. The prevalence of vulvovaginal candidiasis (VVC) is increasing due to the extensive utilization of broad-spectrum antibiotics as well as increasing cases in immunocompromised patients (17). *Candida albicans* is the most common and clinically relevant species that accounts for 85-90% of VVC (25). However, there has been a significant trend towards the emergence of other species such as *Candida glabrata* and *Candida krusei,* which show more resistance to the first line antifungal treatments (30). Because of the fact that VVC strikes millions of women annually, leading to great discomfort, interfering with sexual and social relationships and impairing work performance, it has been considered an important worldwide public health concern (26).

In recent years, the increasing rate of fungal infections has led to an increasing resistance to antifungal agents. Studies show that 7.5% of the patients present resistance to one or more of the commonly prescribed antifungal agents (28).

Moreover, the therapeutic arsenal available for the treatment of fungal infections is quite restricted, being limited to polyenic and azolic antifungal chemicals (11).

In the treatment of VVC the main groups of antifungal agents used are nystatin (cream or vaginal ovule), and azoles (10). The azoles are wide-spectrum drugs, being active against fungi and fluconazole is the most frequently used in the treatment of VVC (9, 28).

Consequently, there is an increasing need to develop standardized in-vitro susceptibility tests that could be used as a model to guide therapeutic decisions and to monitor their effectiveness (6). One of the most widely used commercial methods is the Etest (AB Biodisk, Solna, Sweden). It is based on the use of a continuous concentration gradient of an antimicrobial agent on a plastic strip transferred to an agar medium (22). Many studies, such as those carried out by Van Eldere et al. (31), Marti-Mazuelos et al. (19) and Favel et al. (12) have found a good correlation between the Etest and the micro- and macro-dilution methods recommended by the CLSI (Clinical Laboratory and Standards Institute) (4, 5).

The purpose of this study was to determine the species distribution and the antifungal resistance among yeasts isolated from women with vulvovaginal candidiasis.

MATERIALS AND METHODS

Patients: Vaginal secretion was collected from 130 patients with symptoms suspected to be vulvovaginal candidiasis (VVC), who sought treatment at the out-patient clinic of the University of São Paulo Hospital. The secretions were considered positive when yeast growth was shown.

Yeast identification: All clinical specimens were placed on CHROMagar *Candida* (Biomeriex, Paris, France) and Sabouraud dextrose agar (Difco, Detroit, USA). Cultures were incubated for 72 h at 35 °C. The identification was performed according to the methods recommended by Kurztman & Fell (16).

Differentiation of C. dubliniensis from C. albicans: The differentiation of *C. albicans* and *C. dubliniensis* was performed by polymerase chain reaction, using primers for specific species (CDU2 for *C. dubliniensis* and CAL5 for *C. albicans),* according to the methods described by Mannarelli & Kurztman (18). These tests used *C. albicans* ATCC64548 and *C. dubliniensis* 777.

Etest: The Etest (AB Biodisk, Solna, Sweden) was prepared according to the manufacturer's instructions. The assay medium was RPMI 1640 (Sigma, USA) buffered to pH 7.2 with morpholinepropanesulfonic acid (MOPS) 1.65 M, supplemented with L-glutamine and 2% glucose without sodium bicarbonate (Probac®). Autoclaved bacteriologic agar (Difco Laboratories, USA) at 3%

concentration was added to the RPMI medium, which was then distributed in volumes of 25 mL per plate. The inoculum, prepared in the test tubes, consisted of isolates of *Candida* spp., cultured for 24 hours in Sabouraud dextrose agar and suspended in 3 mL of sterile saline solution with a McFarland turbidity of 0.5. The inoculum was evenly spread over the agar surface with sterile cotton swabs. After a 15 minute drying period, Etest strips were placed on the agar surface and the plates were incubated at 37 °C. MIC endpoints were read according to the Etest instruction manual after 24 h of incubation. The interpretation of the results was based on the MIC values recommended by the CLSI M27-A2 (4) and M27-S3 (5) for amphotericin B, fluconazole, ketoconazole, itraconazole and voriconazole. *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were used as quality control strains throughout all experiments.

Disk-diffusion agar: This test was carried out according to the manufacturer's instructions (Sensifungidisc – Cecon®), for nystatin. The disks used were impregnated with 100 IU of nystatin. The medium used was Müller-Hinton (Difco, Detroit USA) agar plus 2% glucose, complemented with methylene blue for better visualization of the inhibition halo. Preparation of the inoculum and the inoculation procedures were carried out in the same way as for the Etest. After the incubation period, the inhibition halo was read. The interpretation of nystatin zones of inhibition was made in accordance with the Sensifungidisc (Cecon) database, with a halo >10mm classed as susceptible and <10mm classed as resistant. Quality control for disk diffusion testing was performed by using *C. albicans* ATCC 90028 and *C. parapsilosis* ATCC 22019.

Statistical analysis: The percentages and graphs were performed using Excel software.

Ethical aspects: Ethical clearance was obtained from the Ethical Committee of the University of São Paulo in accordance with the Helsinki Declaration of 1975. The patients were recruited and agreed to participate by signing an informed consent form.

RESULTS

Among 130 women studied, 63% (82/130) were confirmed by laboratory tests as positive for candidiasis, 36.2% presented bacterial vaginosis and 0.8% presented trichomoniasis. Among the yeasts isolated, *C. albicans* was the most frequently identified with 87%, followed by *C. glabrata* (7%), *C. parapsilosis* (4%) and *C. tropicalis* (2%). No strains of *C. albicans* were identified as *C. dubliniensis* according to the PCR method. The identified yeasts and their percentage occurrence in the study are shown in Figure 1.

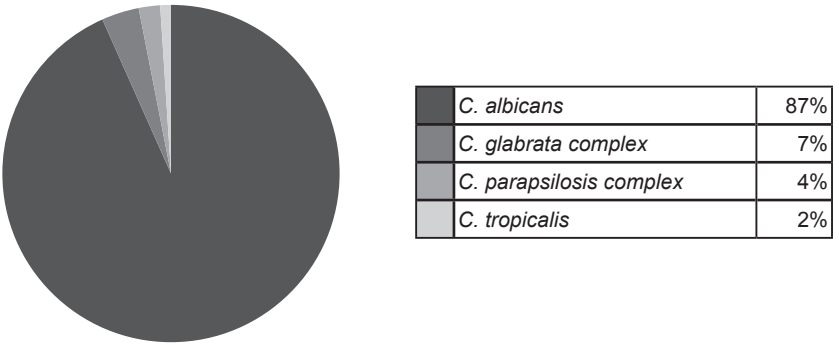

Figure 1. Frequency (%) of yeasts isolated from vaginal secretion of patients with vulvovaginal candidiasis.

The MIC values (MIC_{50} and MIC_{90}) determined by Etest methods for the 82 isolates tested covered a broad range, as shown in Table 1. The trailing phenomenon was observed in 34.1% (28/82) of the isolates for ketoconazole, itraconazole, fluconazole and vorizonazole.

Table 1. Susceptibility in vitro of the 82 strains of *Candida* spp. isolated from vaginal secretion using Etest.

Antifungal Drug	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	MIC Range (µg/mL)
Amphotericin B	0.125	0.25	0.002-1.5
Fluconazole	0.25	0.50	0.064-1.5
Voriconazole	0.0008	0.016	0.002-0.064
Ketoconazole	0.008	0.032	0.002-0.125
Itraconazole	0.047	0.125	0.003-0.38

According to the CLSI Document M27A2 (16) and S3 (17) proposed criteria, 81 (98.8%) of the isolates were susceptible to amphotericin B; the unique resistant isolate was *C. albicans*.

All the isolates were susceptible to ketoconazole according to the CLSI proposed criteria. However, according to Cartledge et al. (20), a MIC ≥ 0.125 µg/mL can lead to therapeutic failure. In the present study one isolate of *C. glabrata* showed a MIC of 0.125µg/mL.

Regarding itraconazole, the CLSI recommends MICs between 0.25 and 0.5µg/mL as Susceptible-Dose Dependent (SDD). By this criterion 97.6% of the isolates were considered susceptible and 2.4% SDD. Among the SDD isolates, one was *C. parapsilosis* and one was *C. albicans.*

According to the CLSI criteria, all the isolates were considered susceptible to fluconazole, although according to the values of Sobel et al. (21), in this study the 2 cases of VVC with a MIC > 1μg/mL may indicate treatment failure. In our study, the MIC values of one isolate of *C. glabrata* and one of *C. parapsilosis* were considered high, placing these strains in the resistant category.

For voriconazole, all the isolates were considered susceptible. All the isolates were susceptible to nystatin.

DISCUSSION

Vulvovaginitis is considered one of the most common complaints encountered in medical practice (15) and vulvovaginal candidiasis (VVC) is the second cause of infectious vaginitis, accounting for between 40% and 50% of all cases (20).

In this study, *C. albicans* was the most frequently isolated yeast species (87%). The species *C. dubliniensis* was not found in our study. Amongst the non-*albicans* species, *C. glabrata* complex is the most frequent followed by *C. parapsilosis* complex and *C. tropicalis*. Similar results were found with other studies, such as those performed by Costa et al. (8) that reported 88.7% of *C. albicans* and 12.3% of non-*albicans* species. In the United States, Richter et al. (24) reported that of 530 samples of *Candida* spp. isolated from vaginal secretion, 70.1% were *C. albicans*, 18.9% *C. glabrata,* 5% *C. parapsilosis* and 6% other species.

Although the incidence of *C. albicans* remained high in the last decade, there was an increase in the prevalence of infections caused by non-*albicans* species especially in chronic cases of vulvovaginal candidiasis. According to Spinillo et al. (29) non-albicans prevalence increased to 70% in a period of 8 years and this may reflect the inappropriate use of antifungal agents, prolonged exposure to these drugs, self-medication and/or incorrect diagnosis of vulvovaginal candidiasis.

In the 1990s the Etest was developed, a commercial kit that allows the determination of a drug's MIC values. Commercial techniques have some advantages over reference methods. Generally, they are easier to perform, are more economical and can be used readily in clinical laboratories (9). Comparative evaluations of Etest versus broth macrodilution and broth microdilution susceptibility testing of various antifungal agents against clinical isolates of *Candida* species indicate that Etest is a promising method for performing antifungal susceptibility testing (21).

Moreover, therapeutic success depends on many factors, including the patient's immune response, the concentration of the drug in the blood, protein breakdown (7), inadequate drug penetration at the infection site (13), interactions with other drugs (22) and, in cases of VVC, the vaginal microbiota (26), as well as factors related to microorganisms, such as the production of biofilms and other virulence factors, including the production of proteinases, which for *Candida* spp.

isolates has a considerable impact on therapeutic success (3). The MIC values defined by the CLSI in document M27 S-3 (5) do not include these aspects.

The present study involved an analysis of susceptibility and resistance based on these criteria and others found in the literature (27), which consider the values of the maximum concentration that the drug reaches in the mucosa (oral or vaginal).

We found a single resistant isolate (*C. albicans)* to amphotericin B, whilst according to the values proposed by Clancy & Nguyen (3), 5 (6.2%) of the isolates, all of which were *C. albicans*, were resistant.

For itraconazole the studies found in the literature do not contest the values given by the reference method. However, there have been discrepancies between in vitro and in vivo results. Burgess et al. (1) observed that isolates that were SDD in vitro showed therapeutic failure in vivo, while Costa et al. (24) obtained a 100% cure rate for in vitro SDD yeasts of vaginal origin.

Fluconazole, in cases of candidiasis, is presently the most frequently used antifungal, being also used prophylactically in recurrent VVC (26). According to the MIC values proposed by the CLSI, all the isolates were susceptible to fluconazole; the MIC values were high as compared for others antifungal agents. Moreover, according to Sobel et al. (27), isolates with a fluconazole MIC over 8 μg/mL should be clinically resistant to conventional doses of fluconazole. In vitro susceptibility testing has not been validated and is not reliable in predicting clinical response in vaginitis. By this value, two *C. albicans* isolates, one *C. glabrata* isolate and one *C. parapsilosis* isolate were resistant. Among the 4 resistant isolates, 3 presented resistance to one other azole tested (itraconazole or ketoconazole). The *C. glabrata* isolate that was resistant to fluconazole also presented resistance to ketoconazole.

Amongst the new triazole antifungal agents, voriconazole has a wider spectrum than itraconazole and fluconazole, and can also be used in the treatment of cases involving species intrinsically resistant to fluconazole, such as *C. glabrata* and *C. krusei*, Moreover, because of the high cost of this medicine, it is not regularly used in cases of VVC. In the present study, it was demonstrated that all the isolates were susceptible to voriconazole, and the MIC values, ranging from 0.002 to 0.064μg/mL, can be considered low if compared with the values obtained for fluconazole (0.064–1.5 μg/mL); this new azole which may prove of therapeutic value in the future.

In cases of VVC, nystatin is the polienic compound most frequently used. However, it is not available by the Etest method and so the agar disk diffusion method was used in this study. All the isolates were considered susceptible. The literature contains few studies on in vitro susceptibility to nystatin.

This study contains relevant information about the diversity of yeast species and antifungal drug susceptibility in relation to the yeast strains present in cases of VVC and may help the clinician choose the most adequate therapeutic orientation.

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DISCLOSURE

The authors declare that there is no conflict of interest.

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