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VULVOVAGINAL CANDIDIASIS IN MATO GROSSO, BRAZIL: PREGNANCY STATUS, CAUSATIVE SPECIES AND DRUGS TESTS

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ABSTRACT

Causative agent in majority of VVC is *Candida albicans,* but infection due to non-*C. albicans* is common. Use of empiric antifungal therapy in Brazil due to syndromic management of vulvovaginitis could act as risk factor for increase resistance among VVC causative agents. From Mato Grosso patients, 160 with culture-proved among 404 women who had clinical symptoms of VVC, were enrolled in this study. 70 non-pregnant women and 90 pregnant women were included. *Candida albicans* was the most prevalent, representing 72.9% in the non-pregnant group and 92.3% in the pregnant group. Differences in species distribution were noted between the two groups, being *C. parapsilosis* the second more prevalent species among non-pregnant women. Susceptibility testing revealed high susceptibility to fluconazole (except for *C. krusei*), itraconazole, ketoconazole, and amphotericin B regardless the species (*C. albicans, C. parapsilosis, C. tropicalis, C. glabrata, C. krusei*) analyzed.

Key words: Vulvovaginal candidiasis, antifungal susceptibility testing, antifungal agents, *Candida albicans*

INTRODUCTION

Vulvovaginal candidiasis (VVC) is a substantial cause of lower genital infections in women, especially in developing countries and cause significant loss of work hours and significant financial burden on the already weak economy. An estimated two thirds of women will suffer at least one episode of VVC in their lives and 40-50% may experience multiple episodes (24). The actual incidence of VVC is unknown since it is not a reportable disease in our country. Vulvovaginal candidiasis is also an important cause of morbidity in the pregnant population, and may contribute to increased healthcare expenditure. During normal pregnancy candidiasis is frequently encountered without significant risk for the fetus. Nevertheless, VVC may occasionally jeopardize an otherwise successful pregnancy. VVC could represent a risk factor for candidemia in preterm neonate during the normal partum. Early detection, early diagnosis and appropriate treatment may

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improve the women, pregnant or non-pregnant, and neonate clinical conditions (17).

Recommendations for vulvovaginitis diagnosis included speculum examination, and microscopic examination of slide from vaginal exudates to look for protozoan (*Trichomonas vaginalis*), bacterial (*Gardnerella vaginalis*) or fungal (*Candida* spp.) agents, followed by vaginal fluid specimen culture to confirm the diagnosis. Otherwise, the confirmatory diagnosis based in culture is not routinely performed nor generally advised in many regions, because all procedures are expensive and time consuming. (3).

Several facts account for not perform routine cultures and antifungal susceptibility procedures in developing countries, like Brazil, for VVC cases. Antifungal susceptibility testing is usually not warranted for individual treatment guidance. Culture procedures are expensive and time consuming thus lead to a delay in the treatment, and there is a risk of loss of patients on follow-up. Identifying Candida by culture in the absence of a positive result of wet preparation from vaginal discharge is not an indication for treatment because approximately 10%–20% of women harbor *Candida* spp and other yeasts in the vagina. (4).

In practice, many Brazilian women who self-diagnosed for immediate and personal treatment of presumed vulvovaginal candidiasis use intravaginal preparations of butaconazole, clotrimazole, miconazole, tioconazole, and boric acid in gelatin capsules, since these medicines are available over-the-counter (OTC). Furthermore, growing sales of generic versions of branded medicines have encouraged pharmaceutical companies to compete on price, and generic products (e.g. oral fluconazole) are usually 35% to 60% cheaper and have ready access. Interesting, previous findings showed many women who self-diagnose and use an over-the-counter product for treatment of presumed vulvovaginal candidiasis do not have vulvovaginal candidiasis (9).

According to some previous studies, the increased use of over-the-counter antifungal drugs and prolonged treatments for recurrent candidiasis are risk factors for the emergence of azole resistance among *C. albicans* isolated from vulvovaginitis patients (22).

All these features urge for monitoring by reference methodologies the relative species distribution and the frequency of resistant isolates that remains concerns to be addressed in studies all over the word. Trends in spectrum and antimicrobial susceptibility of yeast species causing vulvovaginitis represent an interesting issue (11). In contrast with North American and European data, little knowledge in this subject is available from Latin American regions. Antifungal susceptibility testing plays an important potential role in the development of regional antibiogram to aid empiric selection of antifungals.

The objective of this study is to investigate species distribution and antifungal susceptibility pattern of yeasts causing vulvovaginal candidiasis in Brazilian pregnant and non-pregnant women.

The work was submitted to the Ethics in Research Committee (*Comitê de Ética em Pesquisa*, CEP) of the General University Hospital (HGU) of the University of Cuiabá (UNIC) prior to its realization; approved protocol CEP: 0305-198.

MATERIALS AND METHODS

Vaginal yeast cultures were collected from patients, pregnant and non-pregnant women, with vaginal discharge, suggestive of vulvovaginal candidiasis. Briefly, from November 2005 to November 2006, the total of 404 subjects, attended at the General Public University Hospital (gynecology obstetric reference in Cuiabá - Mato Grosso State), Brazil, were studied. A sample from vaginal fluid was obtained with a sterile cotton-tipped swab according the decision to submit a culture made by the attending gynecologist. All speciments were inoculated onto Sabouraud dextrose agar (Difco) and incubated for 24-96 h at 35°C in ambient air atmosphere.

Subsequently the positive cultures were plated on CHROMagar Candida (BBL) to ensure detection of mixed infections. Identification was based on colony morphology and carbohydrate assimilation using standardized auxanographic and zimogram methodologies besides cornmeal agar morphologic analysis (7). The isolates were banked in glycerol 15% at -20°C temperature.

Susceptibility testing was performed using a broth microdilution method according to the reference method M27-A2 (6). Antifungals and concentrations tested were: fluconazole (Pfizer Laboratories, Br) (0.12 to 64 µg/ml), itraconazole (Janssen – Cilag, Br), ketoconazole, and amphotericin B (Sigma, St. Louis) (all 0.015 to 8 µg/ml).

The initial inoculum was prepared from the dilution of the cell mass collected aseptically with a sterile loop in 5 ml of sterile saline solution 0.85%. Turbidity was compared to the McFarland Standard no. 0.5, at a concentration of 1 to 5 x 10^6 cells/ml (6). A 100 µL final yeast inoculum of 1 to 5 x 10^3 cells/ml in RPMI 1640 (Sigma Chemical, St. Louis) medium was added to each microdilution well. The trays were incubated at 35ºC in a BOD incubator for 48 h. The MICs were read as the lowest antifungal concentration with substantially lower turbidity (50%), compared to growth in the antifungal-free growth control well for all azoles. For amphotericin B, the MIC value was defined as the lowest concentration with complete inhibition of growth. CLSI (6) interpretative criteria were applied for the azole drugs. For amphotericin B MIC > 1µg/mL were typically for resistant isolates.

Quality control was ensured by testing the CLSI-recommended quality control strains of *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258.

RESULTS

Among 404 studied women presenting vaginal discharge, 160 patients were diagnosed as having VVC in a total prevalence (IC 95%) for vulvovaginal candidiasis of 39.6% (35% - 44%), including 34.5% (28% - 41%) non-pregnant women and 44.8% (38% - 52%) pregnant women. The group age ranged from 16 to 50 years-old. Of the 160 patients with VVC diagnosis, 27 were asymptomatic presenting only vaginal discharge, 133 had two or more symptoms.

The species obtained from non-pregnant patients were distributed as follows: 51 (72.9%) were identified as *C. albicans*, 10 (14.3%) as *C. parapsilosis*, 4 (5.7%) as *C. tropicalis*, 3 (4.3%) as *C. glabrata*, and 2 (2.8%) as *C. krusei*. Of the species obtained from pregnant patients, 83 (92.3%) were identified as *C. albicans*, 3 (3.3%) as *C. krusei*, 2 (2.2%) as *C. glabrata*, 1 (1.1%) as *C. parapsilosis*, and 1 (1.1%) as *C. tropicalis*.

Tables 1 and 2 represent the ranges of the minimal inhibitory concentrations (MIC), as well as the MIC_{50} and MIC_{90} values obtained for the nonpregnant group (n = 70) and the pregnant group (n = 90), respectively. The susceptibility tests for the 160 isolates revealed that resistance to azoles was not detected, with the exception of *C. krusei* isolates, intrinsically resistant to fluconazole. Only one of 3 isolates of *C. glabrata* obtained from non-pregnant patients, showed susceptibility-dose-dependent (S-DD) to itraconazole (MIC 0.5 µg/mL). All 160 Candida isolates were ketoconazole and amphotericin B susceptible.

Table 1. Variations in minimal inhibitory concentration (MIC), MIC_{50}, and MIC_{90} for isolates of *C. albicans, C. parapsilosis, C. tropicalis, C. glabrata*, and *C. krusei* in nonpregnant patients, for four antifungal drugs, determined by CLSI testing.

Samples and Antifungal Drugs	CLSI		
	Range (µg/mL)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)
***C. albicans* (51)**			
Ketoconazole	0.015 – 0.25	0.015	0.015
Fluconazole	0.12 – 1.0	0.25	0.50
Itraconazole	0.015 - 0.12	0.03	0.06
Amphotericin B	0.015 - 0.5	0.06	0.12
***C. parapsilosis* (10)**			
Ketoconazole	0.015 - 0.03	0.015	0.03
Fluconazole	0.12 - 2.0	0.25	1.0
Itraconazole	0.015 - 0.06	0.015	0.03
Amphotericin B	0.015 - 0.12	0.03	0.12
***C. tropicalis* (4)**			
Ketoconazole	0.015 - 0.015	0.015	-
Fluconazole	0.12 - 0.5	0.12	-
Itraconazole	0.015 - 0.06	0.015	-
Amphotericin B	0.12 - 0.12	0.12	-
***C. glabrata* (3)**			
Ketoconazole	0.015 - 0.25	0.06	-
Fluconazole	0.12 - 4.0	0.12	-
Itraconazole	0.015 - 0.25	0.06	-
Amphotericin B	0.015 - 0.25	0.03	-
***C. krusei* (2)**			
Ketoconazole	0.06 – 1.0	-	-
Fluconazole	2.0 - 64	-	-
Itraconazole	0.25 - 0.25	-	-
Amphotericin B	0.015 – 0.5	-	-

Table 2. Variations in minimal inhibitory concentration (MIC), MIC_{50}, and MIC_{90} for isolates of *C. albicans, C. krusei*, and *C. glabrata* in pregnant patients, for four antifungal drugs, determined by CLSI testing.

Samples and Antifungal Drugs	CLSI		
	Range (µg/mL)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)
***C. albicans* (83)**			
Ketoconazole	0.015 - 0.25	0.015	0.03
Fluconazole	0.12 - 1.0	0.25	0.25
Itraconazole	0.015 - 0.12	0.03	0.06
Amphotericin B	0.015 - 0.25	0.06	0.12
***C. krusei* (3)**			
Ketoconazole	0.12 - 0.25	0.12	-
Fluconazole	0.12 - 1.0	0.25	-
Itraconazole	0.06 - 0.25	0.25	-
Amphotericin B	0.015 - 0.5	0.25	-
***C. glabrata* (2)**			
Ketoconazole	0.015 - 0.03	-	-
Fluconazole	0.12 - 2.0	-	-
Itraconazole	0.03 - 0.03	-	-
Amphotericin B	0.12 - 0.12	-	-

DISCUSSION

In general, the literature data shows similar figures to those found in the present study oscillating between 28% to 66%, varying according to the population size and the percentages found for non-*C. albicans* species when identification was performed. In our study we have observed among 404 women showing lower genital infection diagnosed as vaginitis on clinical examination, only seventy non-pregnant and ninety pregnant women presenting vaginal discharge with microbiologic diagnostic of VVC *Candida* spp accounted for 39.6% of cases similar to an extensive Austrian research (30.4%). In a Brazilian study, Simões *et al*. (23) described the total prevalence of infection was 40.6% among women with vaginitis, in which Candida species account for 19.3% cases. In Jamaica, Kamara *et al*. (13) evaluated 269 pregnant women, reporting a prevalence of 30.7% for candidiasis, a value surpassed only by that for bacterial vaginosis (44.1%). Ashraf *et al*. (2) realized out a study with the purpose of determining the correlation between vaginitis symptoms and candidiasis and the prevalence of *C. albicans* in pregnant women. Of a total of 262 women, 74 cases were positive in culture, with subsequent identification of the species *C. albicans*.

The percentage found for the species *C. albicans* (90.4%) in the article published by Garcia Heredia *et al*. (10), was similar to that found in this study (92.3%) for pregnant patients. The same prevalence of *C. albicans* in relation to the other species is described in other articles (10, 18, 27). However, Us *et al*. (27) studied a group of 218 pregnant women described signs and symptoms of vulvovaginal candidiasis, observed the percentage of 53.2% for the species *C. albicans*, which is a much lower figure than that found in the present study. Conversely, in some studies VVC was found in very low rates (8%-15%) among women showing vulvovaginitis contrasting with others reports (1, 14).

The role of *Candida* species as a cause of vulvovaginitis is controversial and as many as 15%-20% of female with vaginal infections are asymptomatic with exclusive vaginal discharge. The absence of rapid, simple, and inexpensive diagnostic tests continues to result in both overdiagnosis and underdiagnosis of vulvovaginal candidosis (26).

We found similar rates of occurrence of VVC in pregnant (44.8%) and non-pregnant (34.5%) although pregnancy is thought to be a risk factor of fungal infection. Furthermore, no correlation was observed among pregnant status and yeast species. Among non-pregnant patients, 51 (72.9%) were identified with *C. albicans*, while 83 (92.3%) were identified among pregnant patients. Secondly, we observed *C. parapsilosis* was identified in 10 (14.3%) non-pregnant patients, as opposed to *C. krusei*, which was identified in 3 (3.3%) pregnant patients. In opposite, pregnancy status of patients displayed a highly significant association with *C. albicans* species whereas non-*albicans* species had a markedly higher prevalence in non-pregnant patients in a recent study (5).

In the present study, the most prevalent species associated

with vulvovaginal candidiasis was *C. albicans* (83.8%) as referred in the literature. *Candida albicans* was the most prevalent cause in 87.9% of an epidemiologic study of VVC cases (19). Several papers indicate the predominance of the species *C. albicans* in vulvovaginal candidiasis episodes; however, other species appear to be increasingly associated with recurring episodes (18). *Candida albicans* is able to adhere to vaginal epithelium more readily than other *Candida* species, which is probably why it caused about 73% and 92% in non-pregnant and pregnant women, respectively, in this study.

Species non-*C. albicans*, as *C. tropicalis* and *C. glabrata* have become increasingly prevalent in some studies, conversely our findings in which these latter show low frequency (20). Data obtained more recently by Ritcher *et al*. (22), reveal a total of 593 yeast isolates from 564 vaginal secretions, with 27% the non-*C. albicans*. Of 429 women that presented positive cultures, 84 (19%) showed multiple positive cultures (233 isolates) during the study. The non-*C. albicans* species were more commonly isolated from patients that showed multiple positive cultures and the predominant species was *C. glabrata*.

Wenjin e Yifu (28) detected a total of 50 isolates in 628 pregnant women, of *C. glabrata*; thus, the prevalence of *C. glabrata* during gestation was 7.96%. Data on the prevalence of Brazilian *Candida* species causing vulvovaginitis remains scarce since cultures are rarely performed. Moreover there are limited data regarding the antifungal susceptibility of yeast causing vulvovaginal candidiasis (VVC) in latin-american countries, particularly, Brazil.

Lopes Consolaro *et al*. (16) evaluated 161 patients, reporting 21.7 % of positivity for VVC. The main causative species was *C. albicans* in 60% of cases. Percentages referring to non-*C. albicans* species were as follows: 27.5% *C. glabrata*, 5.7% *Sacharomyces cerevisiae,* and 2.9% of *Trichosporon* spp.

Development of resistance by *C. albicans* is described almost exclusively in strains of mucosal disease in AIDS after long-term therapy with fluconazole. A recent study investigated antifungal susceptibility and genotypes of *C. albicans* strains from patients with VVC and concluded that *C. albicans* genotype B from patients with VVC was more resistant to itraconazole (15). Distinct genotypes are present in a single isolate that can explain differences in resistance rates reported in *C. albicans* strains. Ribeiro *et al*. (21) studied the expression of the MDR1, CDR1, CDR2 and ERG11 genes in Brazilian *C. albicans* isolates from VVC. They found that the strains with reduced susceptibility to fluconazole had increased expression of CDR1 when compared with the fluconazole-sensitive strains. Resistance may develop secondary to accumulation of many resistance factors over time in a single strain. These may explain why azole resistance has been relatively slow to emerge among *Candida* spp (8).

Nevertheless, the resistance of *Candida spp* to azole antifungals continues to be a significant problem in fungal infections in same instances in patients receiving prolonged therapy (22). Increased resistance to fluconazole has been reported in oral, esophageal and urinary *Candida* isolates, but this has not been observed commonly in genital tract isolates.

The multicentric study of Sobel *et al*. (25), evaluated 556 women suffering from vulvovaginal candidiasis. Of 393 isolates of *C. albicans*, 377 (96%) were very susceptible to fluconazole, which is a percentage very similar to that obtained in the present study. However, Sobel *et al*. (25) detected MIC values that were equal to or higher than 64 µg/mL in 14 (3.6%) *C. albicans* isolates, which reveals resistance. It is important to stress that those authors performed the susceptibility tests after the day 14 of fluconazole treatment. Thus, 73 isolates were obtained after the end of the treatment, from patients that presented treatment failure and a mycological presence. Their analysis revealed only 4 (5.5%) resistant isolates and one that was dose-dependent susceptible. The 68 remaining isolates remained susceptible. Furthermore, the authors obtained *C. albicans* isolates from 101 patients on day 35 of treatment and

some of the patients had become symptomatic, while the majority remained asymptomatic. Of the 101 isolates, three showed resistance to fluconazole. No dose-dependent susceptibility was observed among the isolates. Considering the remaining 98 isolates, MIC values lower than 1µg/mL were observed in 96 of these, indicating that clinical and mycological recurrence occur in patients infected with isolates that are highly susceptible to fluconazole. As for *C. glabrata* isolates, of a total of 44, 2 (4.5%) were resistant to fluconazole (MIC ≥ 64µg/mL), according to Sobel *et al*. (25). MIC values between 16 and 32 µg/mL were not observed in any of the isolates and for 42 (95%) out of 44, values ≤ 8µg/mL were observed, which are considered susceptible.

Houang *et al*. (12) performed measurement of the levels of fluconazole in vaginal secretion of patients that received a single dose of this antifungal drug equal to 150 mg/mL. The peak concentration of fluconazole in secretions rarely exceeded 2.0 µg/mL. Thus, in the article published by Sobel *et al*. (25), the authors correlated the treatment response to fluconazole using MIC values ≤ 1 µg/mL as susceptible.

According to Sobel *et al*. (25), performing in vitro susceptibility tests for all patients suffering from severe or recurring vaginitis caused by yeasts of the genus Candida is not be recommended. This procedure would only be justified in situations of treatment failure for individual episodes. It is noteworthy that the same authors suggested that cases of vaginitis caused by *C. albicans* isolates resistant to fluconazole remain uncommon.

Questions remain regarding issues such as: why women infected with isolates that are very susceptible in vitro occasionally do not respond clinically, and why many women do get better clinically but remain colonized with *C. albicans* isolates recovered after treatment with fluconazole. In addition, yeasts that show high MIC values tend to persist in the vaginal mucosa (16). In the study of Ritcher *et al*. (22) among of the 593 isolates, resistance to fluconazole was detected in only 3.7% (*C. glabrata* 15.2%), and for itraconazole, resistance was observed in 16.2% of the isolates.

In this study we stressed the prevalence of *C. albicans* as etiologic agent of VVC and the in vitro efficacy of commonly used antifungal drugs against this as well as *C. parapsilosis*, *C. tropicalis* and *C. glabrata* isolates. In our point of view these findings were unexpected since in Brazil vaginal fungal infection is a common and troubling nuisance for many women and thus attempt self-treatment with OTCs meant to eradicate candidal infection. In addition, for more than a decade the symptomatic management approach was instituted all over the country and could be a critical factor in determining increased frequency of resistant causative CVV strains. Our findings mean that an explosive expansion of resistances is unlikely to occur. In a symptomatic management a presumptive treatment with azole drugs for VVC is recommended, based in this reported major causative agent *C. albicans*. However, some physicians or gynecologists might be concerned about the possibility of infection with a less susceptible strain, even if the risk is perceived to be low. The risk of women acquiring an VVC by azole-SDD or azole-resistant *Candida* isolates has not been well studied, but several studies showed low rates of in vitro resistance in this population Indeed, in epidemiologic studies on human and environmental *Candida* spp isolates there is convincing data that azole-resistance is quite uncommon. Furthermore the optimal treatment of non-*C. albicans* VVC remains unknown and the options include longer duration of therapy with a non-fluconazole azole drug as first-line therapy, or new classes of drugs, usually, more expensive and less available at public health-care services.

We emphasized that resistant yeasts associated to VVC, both *Candida albicans* and non-*C. albicans*, are rare and are mainly correlated to the presence of *C. krusei* (3%). These findings are meant to serve as a source of clinical guidance in the context of local disease prevalence. On the other hand, OTC healthcare is expected to see significant growth with demand being driven by Brazilians becoming increasingly informed about what kind of products they should take for

certain ailments. The distribution of medicines is restricted to drugstores and pharmacies, but the number of parapharmacies/drugstores outlets continues to grow, and induces self-treat, with chained grocery retailers investing in opening their own drugstores inside their establishments. Unfortunately, we did not assess usage of antifungal therapy in our patients. Studies to intensively monitor long-term changes in the causative VVC agents receiving antimycotics are imperative.

In summary this study stress that the main causative agent of vulvovaginal candidiasis remains *Candida albicans* (~84%) a well kwon azole-susceptible species and reinforce the idea that the second agent *C. parapsilosis* (~7%), and the other species *C. tropicalis*, *C. glabrata* and *C. krusei* (~3% each one) show no-resistance to fluconazole, except *C. krusei* a intrinsically resistant species. Lower prevalence of itraconazole susceptibility-dose-dependent (S-DD) in a single *C. glabrata* isolate. In addition, all these species from patients with VVC was susceptible to ketoconazole, and to amphotericin B. Nevertheless, take in account routine syndromic treatment for lower genital infections with azole drugs as a cost effective strategy, continuous monitoring for species and antifungal susceptibility profile of VVC agents should be addressed through well-equipped and reference investigative laboratories. Otherwise, decisions regarding which culture for identification and/or antifungal susceptibility testing should be performed must be made on an individual basis. Further research on the consequence of theses approaches in changes in the vulvovaginal candidiasis causative agents is warranted.

In general, we consider that it is necessary to perform a larger number of epidemiological studies not only to evaluate the distribution of the species of yeasts of the genus Candida in cases of vulvovaginitis, but also aimed at recovering isolates in patients posttreatment, especially when treated with fluconazole.

The paucity of predisposing conditions, the diversity among infecting yeasts, and the predominance of antifungal susceptible *C. albicans* isolates support host defect in vaginal mucosal immunity as a key factor in the pathogenesis of recurrent candidal vulvovaginitis (22).

We believe it is relevant to realize cultures with the aim of obtaining non-*C. albicans* species in those patients suffering from recurring episodes of vulvovaginal candidiasis. These species are usually less responsive to azoles and certain species present resistance to these compounds.

On the other hand, empirical treatments with azoles are apparently supported by the low values observed for the azole derivatives for the species *C. albicans*. Therefore, we do not consider it justified to routinely test for in vitro susceptibility in cases of vulvovaginitis. For justifiable cases, in which clinical symptoms, therapeutic failure and frequent recurrence are observed, we consider that it is important to realize species identification and antifungal drug susceptibility in vitro by a commercial method that shows good correlation to a reference method (eg., the E-test), and detailed anamnesis, with the aim of detecting factors predisposing the patient to recurrent vulvovaginal candidiasis.

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